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Cerebroprotective Activity of U-50488h: Relationship to Interactions With Excitatory Amino Acids and Calcium.

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CEREBROPROTECTIVE ACTIVITY OF U-50488H: RELATIONSHIP TO INTERACTIONS WITH EXCITATORY AMINO ACIDS AND CALCIUM.

A DISSERTATION

Submitted to the Graduate Faculty of the
Louisiana State University and Agricultural
and Mechanical College in partial
Fulfillment for the degree of Doctor of Philosophy

in

The Interdepartmental Program in Veterinary Medical Sciences
(Toxicology Option)

by

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ABSTRACT

The mechanism underlying the anticonvulsant and cerebroprotective activity of U-50488H was evaluated using $^{45}Ca^{++}$ uptake in rat Ficoll purified synaptosomes, (^{3}H)-2-deoxyglucose uptake in selected mouse brain regions, (^{3}H)kainic acid binding to mouse forebrain synaptic membranes and incidence of KA-induced lesions in the CA3 region of the mouse hippocampus.

U-50488H causes reduction in K^{+}-evoked $^{45}Ca^{++}$ uptake. These effects are comparable to those of the calcium channel blockers verapamil and nifedipine and seem to be related to calcium dependent mechanisms. Changes in saturability, specificity and dissociation constant values of kainic acid receptor binding were demonstrated in the presence of U-50488H at concentrations similar to those used in $^{45}Ca^{++}$ uptake studies and in the presence of calcium and chloride ions.

The ability of U-50488H to block kainic acid binding was more pronounced on total specific binding than on high affinity binding.

The effects of U-50488H on neuronal metabolism in areas associated with generation of seizures was evaluated. The kappa opiate anticonvulsant did not have pronounced inhibitory effects on cerebral metabolism and it did not block the activation of metabolism induced by the stimulation of kainic acid receptors.

Significant differences were observed in the mean area of vacuolization induced by kainic acid in the CA3 area of the mouse hippocampus when comparing the U-50488H treated versus the neurotoxin treated group when assessed with parametric and non-parametric methods.

The absence of kainic acid-induced vacuolization in the CA3 region of the hippocampus in the presence of U-50488H, and the requirement of calcium and choride ions for U-50488H-increased affinity of KA at excitatory receptors and decreased B_{max} suggest that U-50488H interfered with a sequence of events induced by excitatory amino acids during the course of the neurodegenerative effects induced by seizures.

U-50488H may be of potential value in the elucidation of the mechanisms underlying neuronal cell death after intense depolarization.

INTRODUCTION

Epilepsy is a symptom of central nervous system dysfunction, not a specific disease (Theodore, 1985).

The epilepsies are a family of neurological disorders that have in common a transient, recurrent, self-sustained interruption of normal brain functions and a simultaneous hypersynchronous activation of a large population of neurons in one focal area or generally throughout the brain (Ditcher & Ayala, 1987). Patients with epilepsy often have frequent interictal discharges seen as spikes or sharp waves on the EEG. The seizures are nearly always correlated with abnormal and excessive EEG discharges. Clinical and EEG observations suggest that seizure activity in most patients with epilepsy has a focal or at least a relatively localized origin (Rall & Schleifer, 1980).

In summary, epilepsy refers to many types of recurrent seizure disorders produced by excessive paroxysmal neuronal discharges. It is increasingly apparent that there are many conditions that cause epilepsy and that the possible causes of seizures are numerous.

It was early proposed (Jackson, 1890) and later confirmed in microphysiological studies (Jasper, 1969) that the capacity of some populations of neurons to generate high-frequency synchronous discharges underlies focal cortical epileptogenesis. From these early studies it was postulated that epilepsy existed in two broad forms: focal or partial epilepsy due to a discrete cortical abnormality and generalized or centreencephalic epilepsy due to an abnormality in deep subcortical grey matter (Ditcher & Ayala, 1987). As clinical and

electroencephalographic analysis have drastically improved differential diagnosis, a better recognition of the true epileptic seizures has resulted (Theodore, 1985).

Today, the epilepsies are described in terms of their manifestations on close-circuit-television videotape and electroencephalography, their mode of transmission, their age of penetrance, their age distribution, and their response to antiepileptic drugs (Delgado-Escueta, et al., 1983).

A great variety of hypotheses have been proposed and discussed to explain epileptic activity. The search for a unified theory about the cellular mechanism of epilepsy has been sought with the hope of developing pharmacological means that could be used to suppress the abnormal activity. So far, no single theory provides the common element to explain the epileptic condition.

Epileptic seizures are caused by an abnormal discharge of cerebral neurones which result in some measurable behavioral alteration or subjective sensation in the patient. The causes of epilepsy remain elusive, and may be detected in some patients but not in others. Epilepsy is nevertheless a "symptom complex", which in some patients may be a genetic abnormality (Porter, 1986).

Irrespective of their diversity of origin, seizures are similar in one aspect: during a seizure neurons in the brain fire together in unusual synchrony. The reason why neurons spontaneously begin to fire in abnornal synchrony is not known. A patient with epilepsy has recurrent spontaneous seizures, not just one. Seizures vary widely in duration, intensity, behavioral manifestations, cerebral spreading and sensitivity to drugs. Even between seizures there is often abnormal

electric activity in the brain of the epileptic patient. Current hypotheses include the roles of excitatory and inhibitory synaptic activity, ionic mechanisms that may be independent of synaptic communication between nerve cells, and specific brain structures or circuits (Barnes, 1987). In the case of synaptic activity, it has been hypothesized that specifically timed inhibitory neuronal activity and excitatory activity is required for synchronous firing and the initiation of a seizure (Engel, 1987). Engel proposes that the firing of a small group of excitatory neurons may shut down a larger region of brain activity by stimulating inhibitory neurons that have synaptic connections throughout that region. A secondary excitatory pulse, properly timed, could then produce a synchronous discharge because all of the neurons begin firing at once. Thus, there is increased synchrony within the primary epileptic zone, which then serves to recruit adjacent and distant areas of the brain. According to Prince (1978), it is the suppression of inhibitory events, not their dominance, that leads to seizures. Drugs that enhance GABA-mediated inhibition are anticonvulsants (diazepam) and drugs that block inhibition are convulsants. Cell synchrony has been explained in terms of a change in concentration of certain ions in the space surrounding brain neurons. This change, coupled with or perhaps due to a shrinkage of extracellular space, may induce the transition to seizure. This mechanism would be independent of synaptic transmission among neurons. A drop in calcium levels may increase neuronal excitability by decreasing synaptically mediated inhibition and also by decreasing the inhibitory influence of potassium efflux that is calcium-dependent (Yaari, 1987).

Research in the last 30 years has been concentrated on models of cortical focal epilepsy. Two paradigms that emphasize how cell communication is abnormal in epilepsy are the mirror or secondary focus of Morrel (1979), and the kindling phenomenon of Goddard (1983). The physiological investigations in the context of these two paradigms have revealed long-lasting alterations and enhancement of evoked potentials and suggests that long-term potentiation may involve the same process as kindling. A more recent model of epilepsy is the generation of seizure-like discharges in slices of brain tissue (hippocampus) maintained in vitro. All experimental models of epilepsy are limited in many respects and may resemble only certain aspects of the human disorder. Nevertheless, these experimental models may provide the key to understanding how seizures are initiated and the underlying basic mechanisms during a discharge and between seizures.

A recent revival of the studies of generation, propagation. and control of generalized seizures has shown that subcortical structures are of great importance in the development and propagation of epileptic activity. There seems to be at least one initiation site for seizures in the rat brain, the area tempestus (Miller & Ferrendelli, in press), and at least two policing structures capable of preventing seizures from occurring: the substantia nigra and the anterior thalamus (Gale, 1985). Whether these brain regions are important in the regulation of seizure activity in humans is not yet known. Questions about the cellular events that underlie the control mechanisms for seizure propagation in both local and generalized seizures remain to be answered. The role of GABAhas been emphasized as the most important inhibitory neurotransmitter, of NMDA receptors for excitatory functions

and of norepinephrine and the opioid peptides as modulatory substances, but little is known about how these transmitters express their role on the target structures, or which target structures create the generalized seizures (Ditcher & Ayala, 1987).

Partial (focal) epilepsies are characterized by seizures in which the first clinical and electroencephalographic changes indicate activation of an anatomic or functional system limited to a part of one or both hemispheres (Delgado-Escueta, et al., 1982). The location of the seizure focus determines the clinical presentation of the seizure. Partial epilepsies are the most common form of seizure disorder, occurring in about 67 per cent of all adult patients with epilepsy and in about 40 per cent of all children with epilepsy (Delgado-Escueta, et al., 1982).

Partial seizures in children and young adults are often due to atrophic nonprogressive structural changes resulting from trauma or birth injuries (foci). The focus is the area of maximal spontaneous epileptiform abnormality when activated with methohexital. Biochemical abnormalities in foci removed at neurosurgery have given contradictory results. Van Gelder, et al., (1972) found that contents of taurine and glutamic acid were reduced and that glycine content was elevated in these foci, whereas contents of aspartic acid and of GABA were diffusely low in cerebral cortex of epileptic patients. Data suggesting that an elevated content of glutamic acid may be involved in the increased neuronal firing in some epileptigenic foci has been provided by Perry & Hansen, (1981). Glycine content was markedly elevated in some foci.

The EEG hallmarks of focal epilepsy both in animal models and in human epilepsy are the ictal, or seizure, discharge and the interictal spike discharge (ID).

The mechanisms by which cellular events lead to the intertictal spike are important to our understanding of the pathogenesis and treatment of clinical focal epilepsy. The interictal spike is a prominent extracellular field potential that is associated with large-amplitude, prolonged depolarization shifts in neuronal membrane potential and with bursts of action potentials.

The neuron engaged in seizure activity has an abnormal feature known as "paroxysmal depolarizing shift" (PDS), and is often associated with high-frequency bursts of action potentials, and synchronous discharge of other cells of the same cortical columnar group. The PDS is a sudden depolarization of the membrane potential, which lasts hundreds of milliseconds and usually triggers a series of action potentials on its rising phase. The PDS, as recorded in a single cell, is coincident with the activity of a large group of cells. The synchronous activity of the group is reflected by the extracellularly recorded field potential. The PDS is followed by a hyperpolarizing potential (the post-DS HP) and neuronal inhibition (Johnston & Brown, 1984; Ditcher and Ayala, 1987).

The interaction of factors such as 1) the existence of intrinsic neuron membrane properties that lead to pacemaker activity in specific subsets of neurons; 2) reduction of inhibitory control mechanisms and 3) excitatory synaptic coupling among neurons of the epileptogenic region allow some predictions about the alterations that might give rise to epileptogenesis in humans.

It is well established that direct block of gamma-aminobutyric acid (GABA), a major inhibitory neurotransmitter, causes seizures, and that augmentation of GABA function can protect against seizure activity. There is considerable circumstantial evidence that supports the GABA hypothesis of seizure disorders. In particular, modulatory receptor sites on the GABA receptor-ionophore complex of the post-synaptic membrane have been described for drugs with anticonvulsant activity, including benzodiazepines and barbiturates. It would appear likely that an impairment of GABA-mediated inhibitory transmission or increased glutamate could contribute to the genesis of at least some kinds of seizure disorders (Olsen, 1982).

When incoordination between the GABA system and other neurotransmitter and modulator systems is present, the defect might involve a local brain region, several brain regions, or the entire CNS. A critically placed local incoordination might have drastic reverberations in the whole nervous system, as in the case of generalized seizures arising from focal cortical lesions. With increasing excitatory input, there would be an increased tendency to release pacemaker neurons and abnormal behavior might be released. Major causes of seizures may be the loss of inhibitory GABAergic terminals at the site of focal cortical epilepsy or a disturbance in various other aspects of GABAergic function or excitatory amino acid transmission (Roberts, 1976).

Glial cells can serve as one major restraining influence on the spontaneous activity of neurons by decreasing the intrinsic excitatory levels of neuronal membranes below their spontaneous firing levels. Such restraint could be exerted by removing substances from the

extraneuronal environment in the region of synapses (K^+ or protons), by adding substances to it, or by the diffusion of substances from it or to it in such a way as to shunt the depolarizing ionic currents.

Because glial cells are important in the control of neuroexcitability, the gliosis resulting from injury may in turn be playing an important role in the generation and spread of partial epilepsy. Direct proof has been provided that K^+ homeostasis in brain is at least partly due to an active transport system into glial cells (uptake). A second kind of transport is passive: redistribution by spatial buffering currents. The electrochemical gradient across glial membranes is so close to the equilibrium state for K^+ that this type of transport is possible. Spatial buffering currents are generated when the membrane potential differs from the K^+-equilibrium potential by an unequal distribution of extracellular K^+-concentration along the cell membrane. Local elevation of $[K^+]o$ results in considerable differences between the membrane potential and K^+-equilibrium potential. Oligodendrocytes have a great capacity to buffer K^+ by passive current movement (Kettenmann, 1986). K^+ can then be released to the extracellular space from a glial process from the original source; this has been proposed as a "spatial buffering" (Kuffler & Nicholls, 1976; Orkland, 1969).

At the cellular level much of the communication that takes place between sensory transducer and neuron, between neuron and neuron, and between neuron and effector cell is believed to occur through the extracellular liberation of a substance or a combination of substances that interact with specialized regions of the membranes of neurons or

muscle and gland cells to produce either excitatory or inhibitory effects.

Electrotonic interactions through gap junctions as well as field effects are also of importance in neural information processing. Changes such as intracellular pH, levels of Ca^{++}, and membrane potential may play a role in determining the extent, efficacy, and stability of gap junctions, and the number and type of neurons that are linked at any time.

In the case of an excitatory substance, its action is believed to result in conformational changes in the membranes upon which it impinges, increasing the permeability to cations such as Na^{+} (result of the opening of Na^{+} channels), and in turn decreasing the potential across the membrane (depolarization). Synaptic excitatory effects upon a neuron occur most frequently on dendrites. An inwardly directed Na^{+} current usually is believed to be responsible for most of the observed depolarization of post-synaptic membranes. Increases of free intracellular Ca^{++} that may occur by inward flow or by release from mitochondria during nerve activity activate the opening of K^{+} channels and have other far reaching metabolic effects. The repolarization of the membrane is produced by the outward K^{+} currents, Na^{+} channel inactivation, and the intervention of Ca^{++}- and Mg++-dependent adenosine triphosphatase to restore the Ca^{++} balance. The Na^{+}-K^{+} pump counteracts the voltage-dependent Na^{+} influx during excitation to reduce passive Ca^{++} influx (Roberts, 1984). Thus, the Na^{+} and Ca^{++} channels are important target sites for studying standard anticonvulsant drugs (Johnston & Ayala, 1975) and other new molecules.

A variety of natural toxins, chemicals, and therapeutic drugs have been found to modify the gating kinetics of the Na^+ channels, thereby altering the excitation pattern (Narahashi, 1974). A pivotal role in the maintenance and restoration of ion gradients that are necessary for the regulation of neuronal excitability is played by Na^+ and K^+-dependent adenosine triphosphatase (Askari, 1982).

The control of intracellular Ca^{++} is not well understood. In addition to the $(Na^+ + K^+)$-ATPase, an ecto-Ca^{++}-stimulated ATPase of uncertain function (Trams & Lanter, 1978), and two Ca^{++} transport systems, the calmodulin-sensitive Ca^{++}-ATPase and a reversible Na^+-Ca^{++}-exchange (Siegel, et al., 1981) have been described. The contribution of each of these systems is so far unknown. Recent work suggests that Na^+-Ca^{++}-exchange processes may act at the cell plasma membrane as well as at the mitochondrial outer membrane (Schellemberg & Swanson, 1983).

Defects in transport have been suggested in epilepsy and could involve reduced clearance of extracellular K^+ released from depolarized neurons via passive mechanisms (spatial buffering) or active transport mediated by $(Na^+ + K^+)$-ATPase (Siegel, et al., 1981). Whether changes in ion pumps are the primary cause of epilepsy or a secondary effect resulting from epilepsy remains unclear. An understanding of the relationship between anticonvulsant molecules and the movements of Na^+, K^+, and Ca^{++} will provide some insight into the ionic mechanisms in epilepsy.

The involvement of excitatory amino acids in neurotransmission in the mammalian brain via multiple excitatory receptors is now supported

by electrophysiological, anatomical, and neurochemical evidence (Olney, 1969, 1978; Watkins & Evans, 1981; Shariff, 1984).

Studies of the relationship between the structure of a series of glutamate analogues, their excitatory potency and their dendrosomatotoxic activity have led to the discovery of a number of experimentally useful "excitotoxins"; examples are analogues of plant origin, such as kainate, ibotenate, domoate, quisqualate, and the synthetic compound N-methyl-D-aspartate (NMDA) (Olney, 1978; Watkins & Evans, 1981). The neurotoxic properties of these compounds when injected locally are similar to those of glutamate: initial depolarization followed by severe neuronal degeneration with relative sparing of axons of passage and glial cells, and with much more toxicity towards some neurons than others. This effect, which can lead to complete neuronal destruction, is believed to occur through the interaction of the "excitotoxin" with receptors for an endogenous excitatory transmitter, located on neuronal somas and dendrites, where the primary "excitotoxic" effects occur. It has been implied that the excessive depolarization produced by the excitatory substance is the cause of neuronal cell death (Watkins & Evans, 1981; Olney, et al., 1974; Ben-Ari, 1985).

Kainic acid (KA), 2-carboxy-4-(1-methylvinyl)-3-pirrolidineacetic acid which was isolated three decades ago from the seaweed Digenea simplex, has been used as an ascaricide in Japan (McGeer, 1978), (Figure 1).

Domoic and quisqualic acids, other analogues of glutamate, share the antihelmintic action and were identified as active principles of other biological extracts used for the same purpose (Olney, 1969). KA

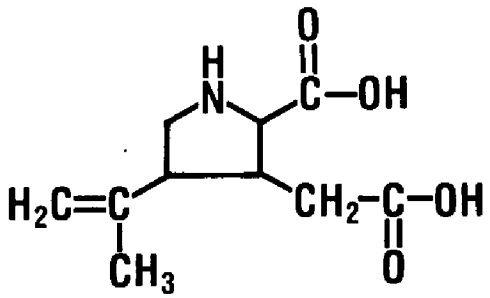


Figure 1. Chemical structure of kainic acid.

is the most potent of these analogues (Coyle, et al., 1984). Parenteral and intracerebral injections of KA produce "axon-sparing lesions", selectively destroying the neurons of a given brain region (Ben-Ari, 1985).

Excitotoxic mechanisms may be responsible for the nerve cell loss in a wide range of neurological diseases including stroke, cerebral palsy, Alzeheimer's disease, Huntington's chorea, epilepsy and other chronic degenerative diseases. Excitatory amino acids may be important in the pathophysiology of seizure disorders. Seizure activity can be initiated by the systemic or focal intracerebral administration of excitatory amino acids. The pattern of KA brain damage and the regional distribution of this damage is reminiscent of that associated with human temporal lobe epilepsy (Schwarcz & Meldrum, 1985; Ben-Ari, 1985).

Intracerebral injections of KA and other excitatory amino acids produce recurrent epileptiform discharge, motor convulsions and damage at the site of injection and in structures distant from the injection site. The distal damage is characteristic of KA and seemingly associated with the limbic status epilepticus and paroxysmal discharges generated by this compound (Ben-Ari, et al, 1980; Ben-Ari, 1985).

The kainate model of limbic temporal epilepsy has been extensively studied. It is particularly useful for the study of the evolution, propagation and pathological consequences of epileptic discharge in the limbic system (Ben-Ari, 1985). Other models of epilepsy have been used such as alumina cream (Gastaut, et al., 1952), cobalt (Mutaris, 1967), zinc (Chung & Johnson, 1983), ouabain (Pealey, et al., 1969), and continuous electrical stimulation of the amygdala in the previously

kindled rat (McIntyre, et al., 1982). The powerful excitatory action of KA has been described in several brain areas (Watkins & Evans, 1981). In vertebrate spinal cord, KA produces a large and often irreversible depolarization which is associated with a reduction in input resistance and depends on the presence of extracellular Na^+ (Evans, 1980). It is possible that the activity of KA is due to an increase in Ca^{++} permeability. This would explain the strong conductance increase and the powerful depolarization, since Ca^{++} carries more current than Na^+ and has a very positive reversal potential because of its low intracellular free concentration (Puil, 1981).

The hippocampus has been the object of numerous studies examining the electrophysiological effects of KA. The effects are particularly more prominent in CA3 pyramidal cells than in CA1. This effect appears to be a synaptic depression, affecting the inhibitory post-synaptic potentials, and, with higher doses, the excitatory post-synaptic potentials. It has been suggested that the action of kainate is at least partially due to a reduction of the GABAergic inhibitory drive. In keeping with this hypothesis, the excitatory phenomena which occur following KA administration can be interpreted as being secondary to decreased inhibition (Evans, 1980).

It has been suggested that the damage inflicted upon the brain by kainic acid has a two-fold etiology: 1) a "local" direct toxic effect at the site of the injection, and 2) a "distant" pathological damage which is related to the epileptogenic effects of the drug. There are major differences between the characteristics of local and distant damage. The former is associated with massive early microglial

proliferation, perhaps of vascular origin. In contrast, glial proliferation in the hippocampus after injection of kainate into the amygdala is not manifested until 4-6 days after the injection, i.e. subsequent to the onset of neuronal damage (Ben-Ari, et al, 1980). It has been postulated that the depolarization produced by excitotoxins will cause "energy-dependent homeostatic mechanisms to draw heavily on the cell's energy stores in an effort to restore ionic balance, with cell death ensuing when energy stores are exhausted or lethal alterations in the composition of the cell's internal milieu have occurred" (Olney, 1978). The administration of anticonvulsants at doses sufficient to prevent the development of paroxysmal discharges fails to prevent the local neurotoxicity of KA. In contrast, anesthesia with gamma-butyrolactone or chloralhydrate-pentobarbital protects hippocampal neurons, suggesting that the anesthetic action plays a role in the protection of these neurons (Ben-Ari, 1985) via interactions with the GABA receptor complex.

The mechanisms of "local" damage remain to be clarified. The local toxic action of kainate may be caused by a complex series of effects, rather than a single mechanism. A possible direct effect of KA on the vasculature of the injected locus remains to be investigated.

The "distant" damage produced in structures away from the injection site or in structures protected by the blood-brain barrier are of two types: The first is selective seizure-related damage, i.e., the damage to CA3 pyramidal neurons. The crucial role of the mossy fibers has been stressed: destruction of the mossy fibers provides immediate protection of the CA3 region against intracerebroventricular KA (Ben-Ari, et al, 1980). Several features of the mossy fiber/pyramidal cell synapse might

cause the selective seizure-related damage of the CA3 pyramidal neurons. Large increases in intracellular Ca^{++} associated with the seizures could exceed the cell capacity to buffer the Ca^{++}, thus leading to an increase in intracellular free Ca^{++} which by means of proteolysis or blockade of the Ca^{++} proton exchange across the mitochondrial membrane may cause neuronal damage (Schanne, et al., 1979). Other factors exclusively localized to the mossy fibers may be released and produce damage during seizures: an endogenous kainate-like chemical (endokain), which will excite the CA3 pyramidal neurons by means of postsynaptic receptors involved in the control of Ca^{++} channels; dynorphin, which is present in large amounts in the mossy fibers and could play a role in this event; an agent released with the transmitter during severe seizure discharge such as Zn^{++} which is particularly concentrated in the mossy fibers and very efficiently blocks Na^{+}-K^{+} ATPase and glutamate decarboxylase (Ben-Ari, 1985). The second type of damage is a non-selective seizure-related damage that can be blocked by administration of anticonvulsants, and is not associated with a specific synaptic connection, best illustrated by a necrosis seen in the pyriform region. This type of necrosis is a lesion typical of anoxic-ischemic damage. It has been suggested that the overactivity caused by KA in this region leads to release of water and metabolites and subsequent edema. Compressing drainage vessels against the skull in the fronto basal region results in a local disturbance of blood flow and anoxic-ischemic changes. This kind of damage is also produced in other brain regions, such as the lateral septum and the CA1 field of the hippocampus (Ben-Ari, et al., 1980; Drejer, et al., 1985).

The kainate model of epilepsy is similar to human temporal lobe epilepsy and fulfills some of the criteria to constitute a useful test model for developing new anticonvulsant drug treatment. The kainate model can shed light on temporal lobe epilepsy in humans and provide a better understanding of the normal and pathological functioning of the hippocampus and other limbic regions.

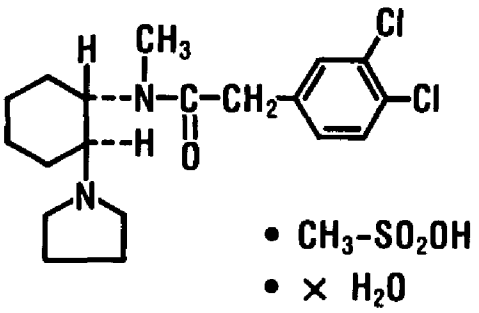
The development of excitatory amino acid antagonists as antiepileptic agents will provide compounds which can block chemically-induced seizures and the neurotoxicity of excitatory amino acids and related analogues.

U-50488H (trans-3,4-dichloro-N-methyl-N-(2-(1-pyrrolidinyl)-cyclohexyl)-benzeneacetamide) (Figure 2) was discovered in the course of the search for an improved analgesic with minimal abuse potential(Szmuszkoviccz & Von Voigtlander, 1982). This compound interacts exclusively with the kappa opioid receptor (Lahti & Von Voigtlander, 1982).

U-50488H is an effective anticonvulsant against electroshock-induced seizures in the rat (Tortella, et al., 1984; 1986; Von Voigtlander, et al., 1986), and produces a dose- and time-dependent suppression of the fully kindled amygdaloid seizure in the rat (Tortella, personal communication). Naloxone at high doses partially antagonizes this anticonvulsant activity, which has a rapid onset and relatively long duration of action.

The effect of U-50488H against electroshock-induced seizures suggests potential efficacy against epilepsies of the generalized or partial types. It has shown an anticonvulsant profile comparable to

U-50488H



Benzeneacetamide, 3, 4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]-, methanesulfonate, hydrate, trans-(±)-

Figure 2. Chemical structure of U-50488H.

that seen with diphenylhydantoin (phenytoin) (Tortella, et al., 1986).

U-50488H blocks the convulsions induced by KA, administered either intravenously or directly into the lateral ventricle in mice, and by the Ca^{++} channel agonist, Bay K 8644. Standard anticonvulsants (phenytoin and phenobarbital) and the calcium antagonist verapamil were ineffective in blocking kainic acid-induced convulsions (Von Voigtlander, et al., 1986). U-50488H at large doses antagonizes the lethal effects of KA and the convulsant and lethal effects of NMDA. This activity may be related to a more general cerebroprotective effect of this compound (Tang, 1985); it is partially antagonized by narcotic antagonists. This suggests that the stimulation of kappa receptors by U-50488H results in protection against seizures and may indicate the possibility that an endogenous ligand for the kappa opioid system is involved in the regulation of neuronal excitability.

I hypothesize that the mechanism of anticonvulsant activity observed with U-50488H is related to the involvement of excitatory amino acid neurotransmitters and Ca^{++} mediated events at the kappa opioid receptor and that the generalized cerebroprotective effect of this compound may be secondary to the anticonvulsant activity.

To test my hypothesis I specifically propose to approach:

i) The biochemical interaction of U-50488H with Ca^{++} in a rat brain synaptosomal preparation and determine the $^{45}Ca^{++}$ uptake at rest and under depolarizing conditions in the presence or absence of this compound.

ii) The excitatory amino acid (EAA) contribution to this anticonvulsant activity by studying the kinetic parameters of a (^{3}H)-kainic acid (^{3}H)KA) binding assay, B_{max} and K_d, with mouse neuronal membranes.

iii) The metabolic changes by determination of (^{3}H)-2-deoxy-glucose uptake (Von Voigtlander, et al., 1983, Sokoloff, et al., 1977) under KA-induced activation in several cortico-limbic areas of the mouse brain.

iv) Finally, to correlate the biochemical and metabolic findings with the morphological changes observed in the mouse hippocampus after KA, the anatomical lesions produced by intracerebroventricular (i.c.v.) injections of KA, and the protective effects of U-50488H on the induction of these lesions quantitated by image analysis.

METHODS

I. $^{45}Ca^{++}$ UPTAKE TECHNIQUES

A. LABELED CALCIUM

1. RADIOLABELED CALCIUM - The ^{45}-calcium chloride [a] in aqueous solution used in all preparations was purchased from Amersham International PLC [a]. The labeled calcium used was from one of several lots having a specific activity of 10-40 millicuries per milligram of calcium at a concentration of 1-5 millicuries/ml of calcium chloride solution.

2. UNLABELED CALCIUM - Calcium chloride dihydrate was purchased from Mallinckrodt, Inc. [b].

B. RAT FOREBRAIN SYNAPTOSOMES.

1. TISSUE COLLECTION AND PREPARATION - Male rats (150-250g) from Charles River Breeding Laboratories [c] were decapitated and the brains quickly removed. Synaptosomes were prepared as follows: dissected forebrain or whole brain was homogenized in ice cold sucrose solution (0.32 M) also containing sodium Hepes (0.5 mM; pH 7.4) (Appendix 1A) (Fleming, et al, 1980). Approximately 1 g of brain tissue was homogenized with 60 ml of the sucrose buffer with a glass-teflon homogenizer (0.15 mm clearance) [d] at 900 RPM (12 strokes), with the tissue maintained in an ice bath (4 C) at all times. The tissue

homogenate was diluted and centrifuged [e] at 3,000 RPM (1,000g) three times for 10 minutes in polycarbonate bottles and the supernatant retained. The pooled supernatants were centrifuged at 20,000 RPM (49,600g) for 10 minutes and the supernatant discarded. The pellet was resuspended to obtain a crude synaptosome preparation which was layered on a Ficoll density gradient [f] made up of 4 ml Ficoll 10%, and 6 ml Ficoll 4% in 0.32 M sucrose (Appendix 1B). Four milliliters of the crude preparation were layered on each tube. The gradients were centrifuged [g] at 13,000 RPM (20,700g) for 40 minutes in a swinging bucket rotor [h].

The cellular material at the interface was collected and pooled. The synaptosomes were centrifuged at 20,000 RPM (49,600g) for 10 minutes. The supernatant was discarded and the pellets resuspended in a 5.6 mM KCl incubation medium (Appendix C), homogenized at 900 RPM (8 strokes) and centrifuged at 20,000 RPM (49,600g) for 10 minutes. The supernatant was discarded and the washing repeated twice. Synaptosomes were equilibrated with incubation medium lacking $CaCl_2$ but containing 5.6 mM KCl (Appendix 1A), at a final protein concentration of 1.5-2.0 mg/ml.

2. PROTEIN DETERMINATION - The protein content of the synaptosome preparation was determined by the Bio-Rad Protein Micro Assay [i], (Bradford, 1976). Dye reagent concentrate (0.2 ml) was added to 0.8 ml of the diluted synaptosomal preparation, then mixed and incubated for 45 minutes at room temperature. Absorbance was read at 595 nm on a spectrophotometer [j] and protein concentration was determined from a

standard curve using bovine serum albumin (range of 1-20 ug) as a standard.

3. MORPHOLOGICAL EVALUATION OF SYNAPTOSOMES BY ELECTRON MICROSCOPY- The integrity of synaptosomes was determined morphologically by electron microscopy after isolation. Synaptosomes were fixed by diluting with an equal volume of 6% glutaraldehyde in 0.2 M phosphate buffer (to a final concentration of 3% gluraraldehyde, 0.1 M phosphate buffer, pH 7.0), and allowed to fix for 1 hour at room temperature. Following three 10 minute rinses in buffer (0.1 M phosphate, pH 7.0), samples were further fixed in 1% osmium tetroxide for 1 hour, then rinsed with three 10 minute buffer changes. Samples were dehydrated with graded ethanols through 100%, rinsed with propylene oxide, and embedded in Polybed 812 [o]. Thin sections (60-80 nm) were cut with a diamond knife on a Dupont-Sorvall MT 5000 ultramicrotome [y], stained with uranyl acetate and Reynolds lead citrate (Reynolds, 1963), viewed and photographed with a Phillips 201 electron microscope [m].

C. $^{45}Ca^{++}$ UPTAKE STUDIES

Synaptosomes were preincubated at 37 C for 10 minutes in calcium-free medium, drug solutions were added, and 1 or 10 minutes later the synaptosomes were loaded with $^{45}Ca^{++}$ for 8 sec. The uptake was initiated by rapidly mixing 0.1 ml of incubation medium (Appendix 2C) supplemented with $^{45}Ca^{++}$ at a specific activity of 0.1 mCi/mMol/L. Uptake was stopped by diluting the samples with 3.0 ml of ice cold incubation medium without added calcium but supplemented with 0.5 mM $LaCl_3$. Extracellularly bound calcium was then displaced by incubation

at 0 C for 20 minutes in the incubation medium containing lanthanum (Gripenberg, et al., 1980).

Synaptosomes were collected by vacuum-filtration on Whatman GF/A filters [k] 3.7 cm in diameter. The synaptosomes retained on the filters were washed with 5 ml (4x) fresh ice cold lanthanum medium (Appendix 1C & 3C) to eliminate the unbound radioactive calcium.

After drying by suction, the filters were placed in counting vials containing 15 ml of ACS scintillation medium [l]. Radioactivity trapped on the filters was measured by liquid scintillation spectroscopy at a counting efficiency of 34%, after the samples solubilized in 15 ml of ACS were kept in the dark overnight to minimize chemoluminescence.

The non-specific binding to filters was determined using incubation medium instead of synaptosome suspension. Control values were determined with 5.6 mM KCl incubation medium (Appendix 1C).

Synaptosomes were depolarized by adding 74.4 mM KCl to the $^{45}Ca^{++}$ solution for a final 40 mM KCl concentration. The filter discs were treated for at least 2 hours in incubation medium supplemented with 2.2 mM $CaCl_2$. This treatment decreased the non-specific binding of $^{45}Ca^{++}$. The incubation medium was prepared daily as were the solutions of $^{45}Ca^{++}$ and drugs. In order to retain isoosmolarity of the medium, the concentration of sodium was decreased when that of K^+ was increased.

Effects of incubation time, K^+ and proveratrine [ac] depolarization, and Na^+ ion absence on $^{45}Ca^{++}$ uptake were determined. In addition the effects of Ca^{++} channel agonists and antagonists, U-50488H, morphine, naloxone, and excitatory amino acids were examined.

II KAINIC ACID BINDING ASSAY TECHNIQUES

A. LIGAND

1. RADIOLABELED LIGAND - The radiolabeled KA [r] used in all binding studies was purchased from Amersham International PLC.
The radioligand had a specific activity of 5.0 millicuries per millimole at a concentration of 1.0 microcuries/microliter of a 2% ethanol aqueous solution.

2. UNLABELED LIGAND - Kainic Acid was purchased from Sigma Chemical Company [s].

B. SYNAPTIC MEMBRANE PREPARATION

1. TISSUE PREPARATION - Charles River CF-1 male mice were decapitated and the brains rapidly removed for preparation of the crude synaptic membrane fraction as described by London & Coyle (1979). This procedure is stated as follows: the tissue was homogenized in 200 ml of glass-distilled water (GDW) at 700 RPM in a Potter-Elvehjem glass homogenizer fitted with a Teflon pestle [d]. The homogenate was dispersed with a Brinkman homogenizer [aa] at setting 6 for 30 seconds. Tissue suspensions were centrifuged [e] at 16,000 RPM (30,590g) for 10 minutes. The membrane pellets were resuspended, recentrifuged, and washed sequentially in 100 ml of Tris buffer (Appendix 2A). The suspension in buffer was incubated for 30 minutes at 37 C to dissociate any glutamate bound to the membranes. After centrifugation for 10

minutes at 16,000 RPM, the pellet was suspended in Tris buffer at 4 C to a concentration of 0.1-0.6 mg protein/ml and used the same day for the binding assay.

2. PROTEIN DETERMINATION - The protein content of the membrane preparation was determined by the Bio-Rad Protein Micro Assay (Bradford, 1976). To 0.8 ml of the diluted membrane preparation 0.2 ml of the dye reagent concentrate [i] was added, mixed and incubated for 45 minutes at room temperature. Absorbance was read at 595 nm on a spectrophotometer [j] and the protein concentration was determined from a standard curve using bovine serum albumin (6 concentrations) within a range of 1-20 micrograms as a standard.

3. MORPHOLOGICAL CONTROL OF SYNAPTIC MEMBRANES BY ELECTRON MICROSCOPY - The homogeneity of synaptic membranes was determined morphologically by electron microscopy after preparation. Synaptic membranes were fixed by diluting with an equal volume of 6% glutaraldehyde in 0.2 M phosphate buffer (to a final concentration of 3% gluraraldehyde, 0.1 M phosphate buffer, pH 7.0), and allowed to fix for 1 hr at room temperature. Following three 10 min rinses in buffer (0.1 M phosphate, pH 7.0), samples were further fixed in 1% osmium tetroxide for 1 hr, then rinsed with three 10 min buffer changes. Samples were dehydrated with graded ethanols through 100%, rinsed with propylene oxide, and embedded in Polybed 812 [o]. Thin sections (60-80 nm) were cut with diamond knives on a Dupont-Sorvall MT 5000 ultramicrotome [n], stained with uranyl acetate and Reynolds lead

citrate (Reynolds, 1963), viewed and photographed with a Phillips 201 electron microscope [m].

C. (^{3}H)KAINIC ACID BINDING ASSAY

1. BINDING ASSAY TECHNIQUE- Aliquots of the crude synaptic membranes were incubated in triplicate at 4 C for 60 minutes in a 2 ml total volume. (^{3}H)Kainic acid ((^{3}H)KA) in a volume of 0.1 ml, various concentrations of the radioligand, displacers and drugs were used. During preparation and incubation, the tubes were kept in an ice-water bath maintained at 4 C. Incubation was prolonged for 60 minutes and terminated by filtration under vacuum with GF/B Whatman filters [q] 3.7 cm in diameter. The filters were rinsed twice with 5 ml of GDW and then counted in 15 ml of ACS solution. Radioactivity trapped on the filters was determined by liquid scintillation spectroscopy of the samples solubilized in 15 ml of ACS at a counting efficiency of 34%. Total specific binding of (^{3}H)KA was defined as the difference between total binding with radioligand alone and non-specific binding measured in the presence of 0.1 mM unlabeled kainic acid (Simon, et al., 1976).

2. SATURABILITY OF SYNAPTIC MEMBRANE KAINIC ACID RECEPTORS- The characteristics of the binding of (^{3}H)KA to synaptic membranes with increasing concentrations of the radioligand were determined by incubation of 1 ml of the final homogenate (0.512 mg protein/ml) for 60 minutes at 4 C as described above in 1C. Radioligand concentrations ranged from 1.25 to 200 nM.

3. (^{3}H)KAINIC ACID HIGH AND LOW AFFINITY BINDING SITES AND IC_{50} CALCULATIONS -

a. To characterize separately the binding to high affinity and low affinity receptor binding sites, advantage was taken of the fact that the radioligand dissociates slowly ($T_{1/2}$ = 90 minutes) from high affinity sites but nearly instantaneously from low affinity sites (London & Coyle, 1979). After 55 minutes of incubation of the membranes with (^{3}H)KA, 200 mmoles of unlabelled kainic acid were added to some experimental tubes to bring the final concentration of unlabeled ligand to 0.1 mM. After 5 minutes of additinal incubation, the tubes were filtered and the binding of (^{3}H)KA was measured.

The addition of 200 mmoles of unlabeled ligand 5 minutes prior to filtration totally displaced the radioligand from low affinity binding sites, but did not affect the non-specific binding; thus the difference between binding in this preparation and those incubated in the presence of radioligand alone represents binding to low affinity receptor sites. The difference between binding occurring in the continuous presence of unlabeled ligand (non-specific) and that occurring with the 5 minute exposure to an excess of unlabeled ligand represents binding to high affinity receptor sites.

b. Specificity of (^{3}H)KA binding to forebrain synaptic membranes was demonstrated by comparing the effects of various analog compounds on the binding of (^{3}H)KA with the membranes. One ml of homogenate was incubated with 40 nM (^{3}H)KA in a total volume of 2 ml, in the same

conditions described above in 1C. Drugs, and ions such as Ca^{++} and Mg^{++} were added to the tubes for competing binding. The filters containing the trapped radioactivity were washed and dried as already described and counted in a liquid scintillation spectrometer [p]. Simultaneous experiments for high and low affinity binding sites were conducted for different concentrations of the drugs used.

4. SCATCHARD ANALYSIS - Linear regression of the data was performed by Scatchard analysis. Estimates of the total number of sites (B_{max}), and the equilibrium dissociation constant (K_d) for the ligand-receptor reaction were determined from the saturation isotherms. The Scatchard program is part of a software package called LIGAND (Munson & Rodbard, 1980).

5. NON-LINEAR ANALYSIS - A weighted non-linear program (Metzer & Weiner, 1984) for one and multiple sites models was used to assess the presence of multiple (^{3}H)KA binding sites.

6. STATISTICAL ANALYSIS- Comparisons of binding data between control and treated groups were made by Student's t-test. Differences were considered significant when $p < 0.05$.

7. INTER- AND INTRA-ASSAY COEFFICIENTS OF VARIATION - To assess the precision of the method, the interassay and the intraassay coefficient of variation were calcutated using standard statistical techiques.

8. DEGRADATION OF THE RADIOLIGAND - To minimize decomposition of (^{3}H)KA solution at 1 mCi/ml, stocks of the solution were stored at 2 C and used as soon as possible after shipment from Amersham [r].

D. DATA OBTAINED - The following parameters were measured from the following (^{3}H)KA experiments:

1. B_{max} and K_d from the following saturation curves:
   a) In Ca^{++} free Tris buffer.
   b) In Ca^{++} free Tris buffer and U-50488H.
   c) In the presence of Ca^{++} ions.
   d) In the presence of Ca^{++} ions and U-50488H.
   e) In the presence of Cl^- ions.
   f) In the presence of Cl^- ions and U-50488H.
   g) In the presence of Ca^{++} and Cl^- ions.
   h) In the presence of Ca^{++} and Cl^- ions and U-50488H.
2. IC_{50} values:
   a) In Ca^{++}-free Tris buffer.
   b) In Ca^{++} free Tris buffer and several concentrations of l-glutamate
   c) In Ca^{++} free Tris buffer and several concentrations of U-50488H.
   d) In the presence of Ca^{++}, Cl^- ions, and U-50488H.
   e) In the presence of Ca^{++} and U-50488H.

III 2-(^{3}H)-DEOXY-D-GLUCOSE UPTAKE IN VARIOUS MOUSE BRAIN REGIONS

A. LABELED 2-DEOXYGLUCOSE

1. The radiolabeled 2-(^{3}H)-deoxy-d-glucose ((^{3}H)DG) used in all uptake studies was purchased from DUPONT* NEN Research Products [t]. The (^{3}H)DG had a specific activity of 7.1 curies per mmole of 2-deoxy-d-glucose at a concentration of 1.0 microcurie/ml of a 1:9 water:ethanol solution.

B. (^{3}H)DG UPTAKE IN VARIOUS MOUSE BRAIN REGIONS

1. ANIMALS - Upjohn CF-1 male mice [c] (18-25 g) were used to determine the uptake of (^{3}H)DG in the hippocampus, corpus striatum and septum (Montemurro & Dukelow, 1972).

2. (^{3}H)DG UPTAKE ASSAY - (^{3}H)DG uptake was evaluated according to the following schedule: saline, KA and/or U-50488H were administered subcutaneously (s.c.) at time zero. Fifteen minutes later 10 microcuries/20 gm of (^{3}H)DG (Appendix 3) in saline was injected intravenously (i.v.) and the distribution and uptake allowed to take place for 30 minutes while the mice were housed in an isolation box supplied with white noise control to standardize sensory stimuli. Individual mice were then decapitated and the brain regions quickly dissected out and weighed in preweighed scintillation vials containing 0.5 ml Protosol [u]. The vials were tightly sealed with Teflon liners [v] and maintained at 50 C overnight.

Each sample was neutralized with 100 microliters of glacial acetic acid per ml of Protosol. ACS (15 ml) [l] was added and radioactivity of the samples was counted with a liquid scintillation spectrometer [p]. The radioactivity was recorded as CPM/mg of tissue.

C. DATA OBTAINED - Uptake was measured from the following groups: a) saline control, b) KA control, and c) KA and U-50488H; data were expressed as both CPM/mg and percent of control.

D. STATISTICAL ANALYSIS- Comparisons of uptake data between control and treated groups were made by Student's t-test. Differences were considered significant when $p < 0.05$.

IV THE EFFECTS OF SUBCUTANEOUS U-50488H ADMINISTRATION ON KAINIC ACID LESIONS IN THE MOUSE HIPPOCAMPUS

A. DRUGS

1. KAINIC ACID - The kainic acid used in this morphometry study was purchased from Sigma Chemical Company [s]. The compound was administered in saline solution at a concentration of 0.04 micrograms/microliter.

2. U-50488H - The compound was generously donated by Dr. Phillip F. Von Voigtlander, The Upjohn Company [w]. U-50488H was used in a saline solution at a concentration of 0.01 gm/ml.

3. ANESTHETIC - Methoxyflurane was purchased from Pitman-Moore, Inc [x].

B. ANIMALS - Charles River CF-1 male mice (18-20 g)]c] were used in this experiment. The mice were housed as groups, with and water and food [ab] provided ad libitum.

C. EXPERIMENTAL DESIGN - The mice were assigned to 4 groups of n= 6-7. Group 1 was a saline control and mice were given saline solution at all times. Group 2 was a positive control and mice received saline and KA. Group 3 was a negative control and mice received U-50488H and saline. Group 4 received U-50488H and KA. Behavior was observed for one hour after the administration of drugs and once daily for the next 4 days. All mice were sacrificed on day 4.

D. PRETREATMENT - The pretreated groups were given saline or U-50488H 100 mg/Kg, s.c. 30 minutes before KA or saline administration.

E. KAINIC ACID INDUCED LESIONS - Mice were given free-hand intracerebroventricular (i.c.v.) injections of 0.08 micrograms KA (total dose) in saline. The mice were allowed to recover for 4 days and water and food (Appendix 4A) provided ad libitum.

F. COLLECTION OF SAMPLES

a. PERFUSION - Animals were anesthetized with methoxyflurane, the chest cavity was opened and saline was injected directly into the heart to flush the circulatory system. Fixation of the brain was accomplished by vascular perfusion of a buffered solution containing glutaraldehyde

and formaldehyde (Karnovsky's fixative) (Appendix 4B) diluted 1:20 with 0.1 M cacodylate buffer, followed by a more concentrated solution of the same buffer.

b. FIXATION - The fixative was delivered rapidly after saline and allowed to flow for 5 minutes. The pressure for the perfusion was kept constant by suspending the bottle containing the fixative and washing out solution at a height of about 60 cm. Care was taken to ensure that no air bubble entered the vascular system. Following perfusion, soft tissues were removed from the outer surface of the skull; the latter was immersed in Karnovsky's fixative for at least 12 hours after removal. The intact brain was removed by careful dissection of the bones of the calvarium; brains were left in fresh fixative for an additional period of approximately 8 days.

G. HISTOLOGY- The cerebellum was removed from the cerebrum with a transverse section through the midbrain at the level of the corpora quadrigemina. Blocks of cerebrum were prepared for embedding with a transverse section at the level of the optic nerve; the cerebellum was sectioned in a similar manner at the level of the pons (Figure 3). Trimmed blocks of brain were embedded in parafin and five serial sections, each 6 micron in thickness, were cut from the trimmed surfaces of each block with a rotary microtome [y]. Resulting tissue sections were stained with hematoxylin-phloxine-eosin. The staining procedure was the same for all batches of slides. The sampling technique was adjusted so that the cytoarchitecture of the hippocampus wasthe same in all slides.

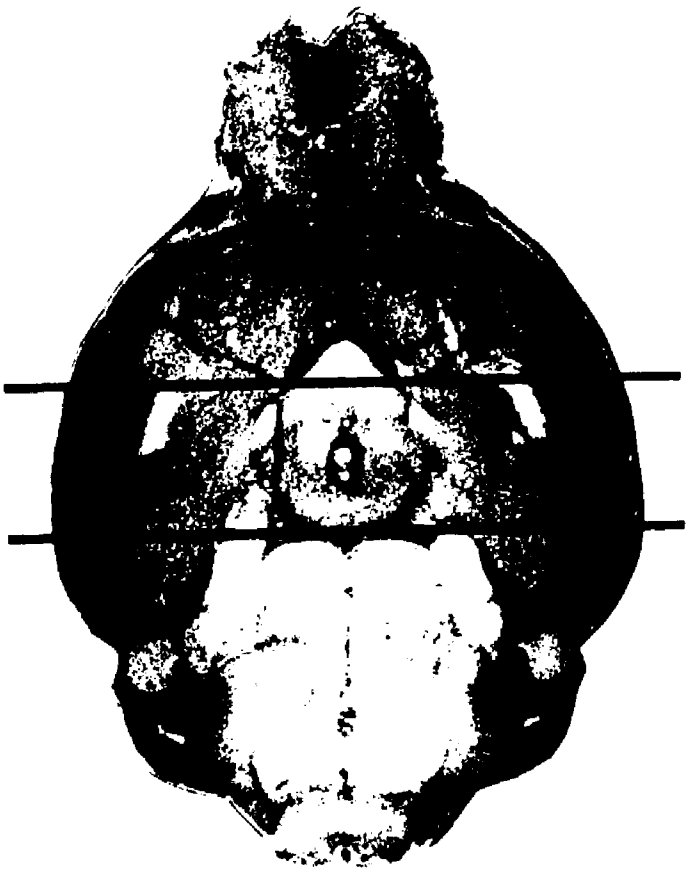


Figure 3. Illustration indicating sites of brain sections taken for histopathological examination.

H. HISTOPATHOLOGY

1. DESCRIPTION OF KA LESIONS - Histopathologic changes were restricted to the neurons and associated neuropil of the granular layer in the CA3 region of the hippocampus. Extensive degeneration and necrosis of neurons resulted in dark, shrunken cell bodies and vacuolization of the neuropil associated with pyknotic neurons.

2. PHOTOGRAPHIC DISPLAY- Fig 4 shows the five levels of the hippocampus where KA lesions were quantitated by image analysis. The effects of 100 mg/Kg U-50488H are illustrated in Fig 5.

I. IMAGE ANALYSIS

1. ADQUISITION OF IMAGES - The video images were acquired using a Magiscan Image Analysis system [z] incorporating a Dage black and white video camera mounted on a Nikon Fluophot microscope fitted with a 20x objective lens. All operations of the image processor were controlled by the computer.

2. IMAGE PROCESSING - The computer digitazed the signal to 6-bit resolution and stored it for image processing. The image processing routine was optimized with a program called "MENU" supplied with the instrument.

Using "MENU" a sequence of grey image and binary operations was performed which conveniently allowed measurement of vacuolized areas. Specifically, it was found that a Gausian transform of the image followed by edge enhancement permitted segmentation of vacuoles from

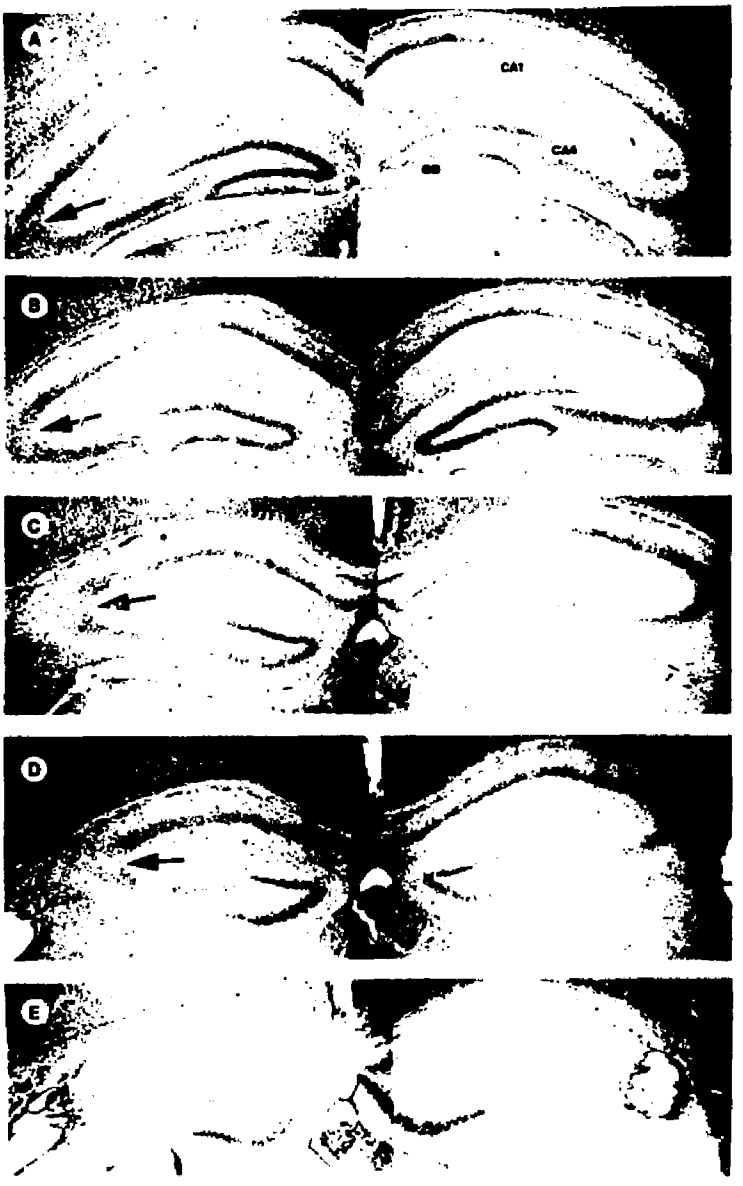


Figure 4. KA-induced lesions (arrows) in the mouse CA3 region of the hippocampus. GD= gyrus dentate; CA1, CA2, CA3 and CA4 = CA1, CA2, CA3 and CA4 regions of the hippocampus. H and E stain. 20X.

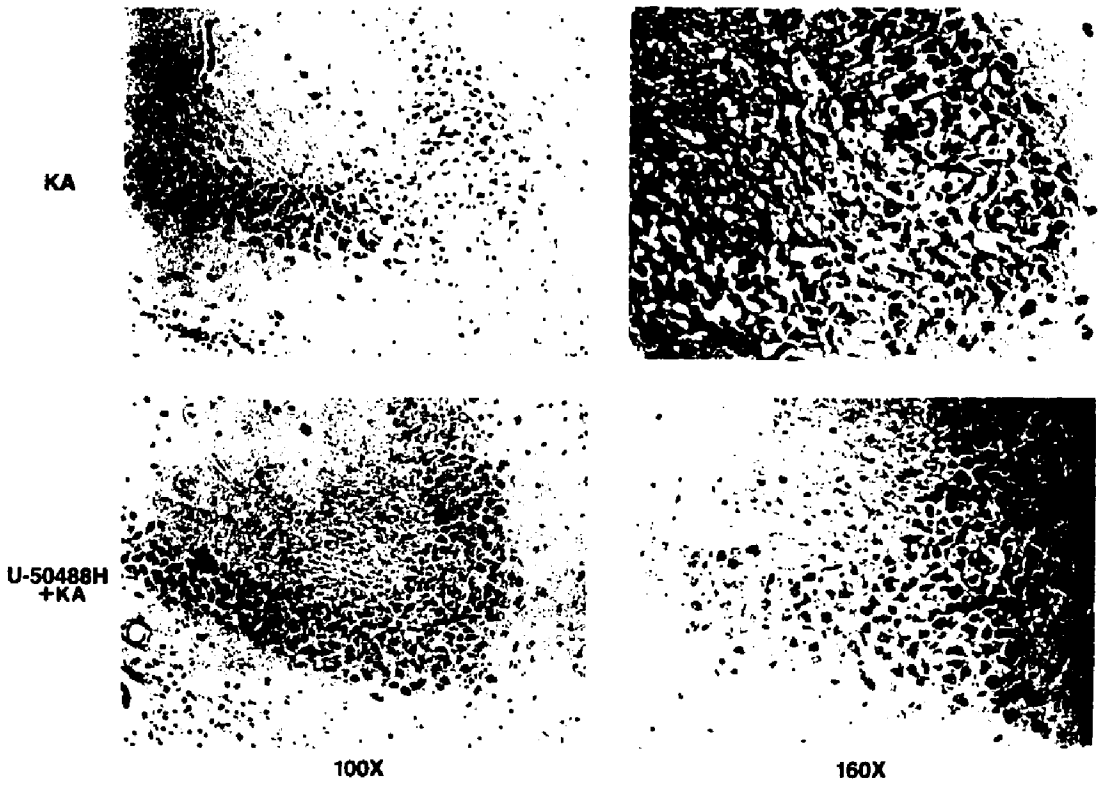


Figure 5. Effect of U-50488H on kainic acid induced lesions in the mouse hippocampus.

surrounding cells. Under normal circumstances no binary image enhancement was necessary. Using this program the area of each vacuole was measured independently and the total area of the field was determined automatically. Interactive editing permitted rejection of unwanted or inappropiate features. Bright regions of the output display showed where there was absence of neuronal cell body (vacuolization) within the granule cell layer of the hippocampus.

The image processing procedure was designed to quantify the area occupied by vacuoles obtained from each slide in a frame of 200x200 pixels (Quantitation), to remove the inappropiate areas such as blood vessels, and artifacts of histological processing, (Editing), to calibrate the measured image (pixels/mm) (Calibration), to subtract unwanted background, and to scale the resulting image in statistically interpretable manner. On control slides, this procedure yielded an image of regional bright area variability, that is, extracellular noise. In the treated animals, this image reflected activity associated with injury to the neuronal cells plus extracellular space. Comparison of the proportion of vacuoles between the different groups revealed those groups in which vacuolization was significantly enhanced or decreased by previously administered KA, drugs or combinations of both.

3. QUANTITATION - For each slide examined, the area of vacuolization in the granule cell layer of area CA3, where most of the lesions were found, was measured with a Joyce Loebl Magiscan 2 system [z] connected to adequate monitoring and recording equipment (ie. microscope, camera and matrix printer). These measurements provided

the basis for the final estimation of the vacuolization profile produced by KA.

J. STATISTICAL ANALYSIS - The data was analyzed by the one-way analysis of variance by pairwise comparison of treatment means using Fisher's Least Significant Difference (LSD). The Kruskall Wallis test was used to analyze the data and to confirm the results of the parametric analysis. P values of <0.050 indicate significat differences between the groups.

RESULTS

I. $^{45}Ca^{++}$ UPTAKE TECHNIQUES

A. SYNAPTOSOMES

1. PROTEIN COMPOSITION OF SYNAPTOSOMAL PREPARATION- The protein concentration of the final synaptosomal suspension was between 1.0- 5.0 mg/ml.

2. MORPHOLOGICAL CONTROL- Examination of the equilibrated synaptosomes by electron microscopy was undertaken to determine the homogeneity and ultrastructural integrity of the cellular material collected. The identifiable subcellular components included mitochondria, morphologically intact synaptosomes, myelin, and unidentified membranous material (Figure 6).

B. $^{45}Ca^{++}$ UPTAKE STUDIES

1. EFFECTS OF TIME, K^+ ION, PROVERATRINE DEPOLARIZATION AND Na^+ ION ABSENCE- Uptake of calcium by the synaptosomes was increased with time in both control synaptosomes and those depolarized by substitution of 40 mM external K^+ on an equimolar basis for Na^+. The time course of this accumulation is shown in Figure 7. For an incubation time of 8 sec, the amount of uptake during depolarization was 1.7 times greater

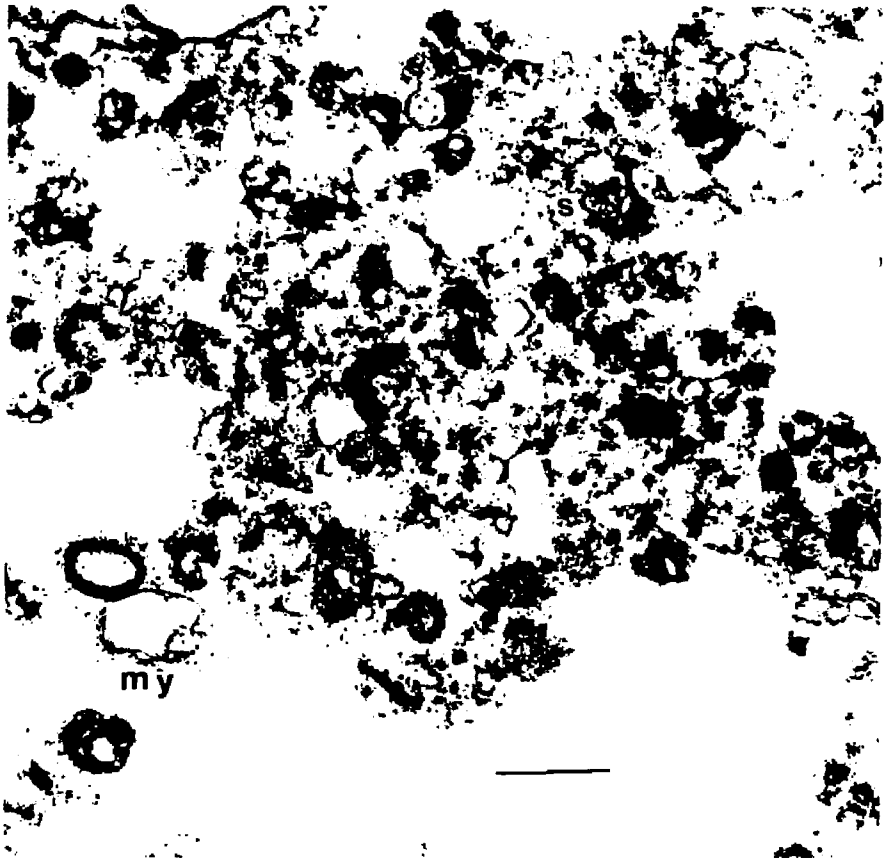


Figure 6. Electron micrograph of rat forebrain synaptosomes prepared on Ficoll gradients. Synaptosomes (S), and myelin (my). Bar = 500um; OsO_4-UL 65,000x.

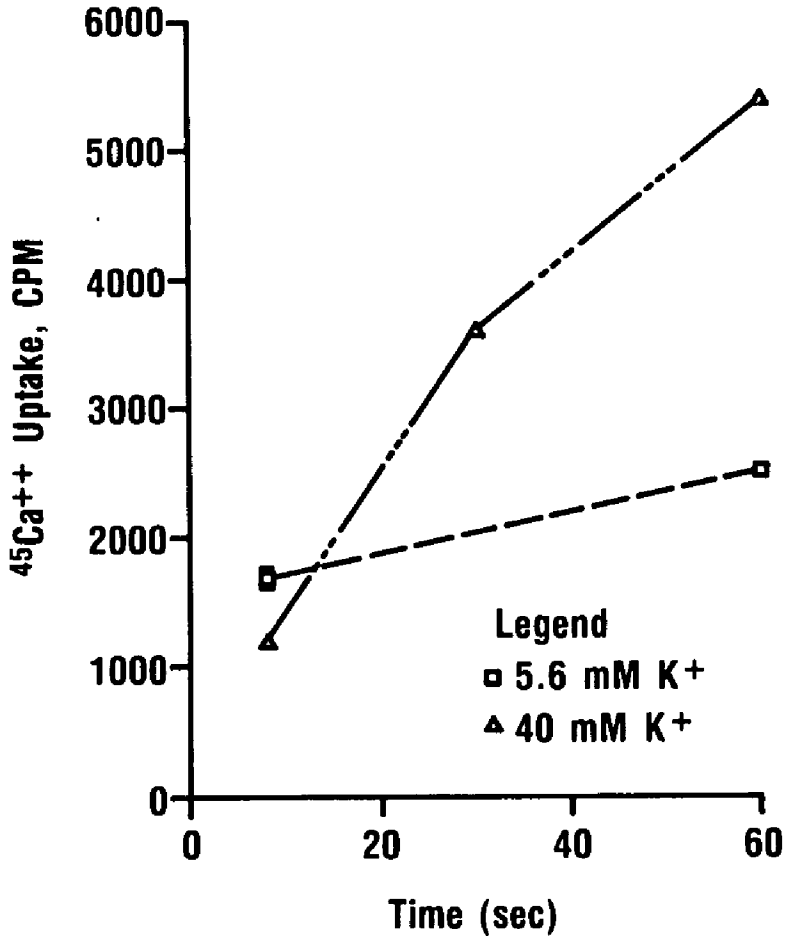


Figure 7. Uptake of $^{45}Ca^{++}$ as a function of time in control (□), and depolarized (△) synaptosomes.

than that in 5.6 mM K^+ buffer. Net Ca^{++} uptake was the difference between control and depolarized uptake. The data in Table 1 indicate that the depolarizing concentrations of K^+ resulted in reliable increases in Ca^{++} uptake. Proveratrine was also able to depolarize the synaptosomes as shown in Table 2. Depolarization has been shown to have an inhibitory effect on Na^+/Ca^{++} exchange (Blaustein et al, 1974). This exchange is electrogenic in nature with respect to Na^+ (exchanging 3 to 4 Na^+ per Ca^{++}) and bidirectional. A reduction of Ca^{++} efflux via Na^+/Ca^{++} exchange has been observed previously as a result of reduction of external Na^+ (Blaustein & Oborn, 1975).

To eliminate the Na^+ dependent processes contributing to the Ca^{++} accumulation, the Ca^{++} uptake was measured using a buffer in which choline was used to substitute for external sodium (Appendix 1C). In a Na^+-free buffer the Ca^{++} uptake of depolarized synaptosomes at 8 sec was not increased when compared to the Na^+ control (Figure 8), (Table 3A). The time course showed that at 60 sec the net uptake of depolarized synaptosomes was the same as that of the K^+ control (Figure 8).

In the presence of external Na^+ (136 mM) the net Ca^{++} uptake of the depolarized synaptosomes at 8 sec was 3.8 times that of the control at 60 sec (Table 1A).

The uptake kinetics of $^{45}Ca^{++}$ have been previously described as having two phases, fast and slow, kinetically characterized by Gripenberg et al, (1980), and Blaustein (1975). The difference between these two phases is the decrease in the

Table 1. Effects of Time and K^+ Depolarization on $^{45}Ca^{++}$ Uptake by Synaptosomes.

	$^{45}Ca^{++}$ uptake CPM(+/-S.D.)		Net Uptake(CPM)
	Control(5.6mM K^+)	Depolarized(40 mM K^+)	
Incubation Time (sec)			
8	1684+/-77	2884+/-289*	1200
30	ND	5283+/-328**	ND
60	2512+/-22*	7068+/-395**	4556
		Prior Depolarization	
8 Control	632 +/- 93	361 +/- 18**	
8 Blank	167 +/- 19	115 +/- 8	

* p <0.01 compared to control (5.6 mM K^+). n=3.
** p <0.01 compared to control (40 mM K^+). n=3.
ND= not determined

Table 2. Effect of Proveratrine on Ca^{++} Uptake by Rat Brain Synaptosomes.

A. Effect of proveratrine in 5.6 mM K^+.

	$^{45}Ca^{++}$ uptake CPM(+/- S.D.)
Control[a]	1690 +/- 131*
Proveratrine 10^{-5}M	2328 +/- 182**
Proveratrine 10^{-6}M	1781 +/- 192
Proveratrine 10^{-7}M	1617 +/- 75

a K^+ 40 mM control= 3989 +/-241 CPM(+/-S.D.).
* $p<0.01$ compared to 40 mM K^+ control. n=4.
** $p<0.01$ compared to 5.6 mM K^+ control. n=4.

B. Effect of proveratrine on depolarized synaptosomes.

	$^{45}Ca^{++}$ uptake CPM(X+/- S.D.)	
	K^+ 5.6 mM	K^+ 40 mM
Control	861 +/- 43	1656+/-139*
Proveratrine 10^{-5}M	1076 +/- 52*	1901+/-191**

* $p<0.01$ compared to 5.6 mM K^+ control. n=4.
** $p<0.05$ compared 40 mM K^+ control. n=4.

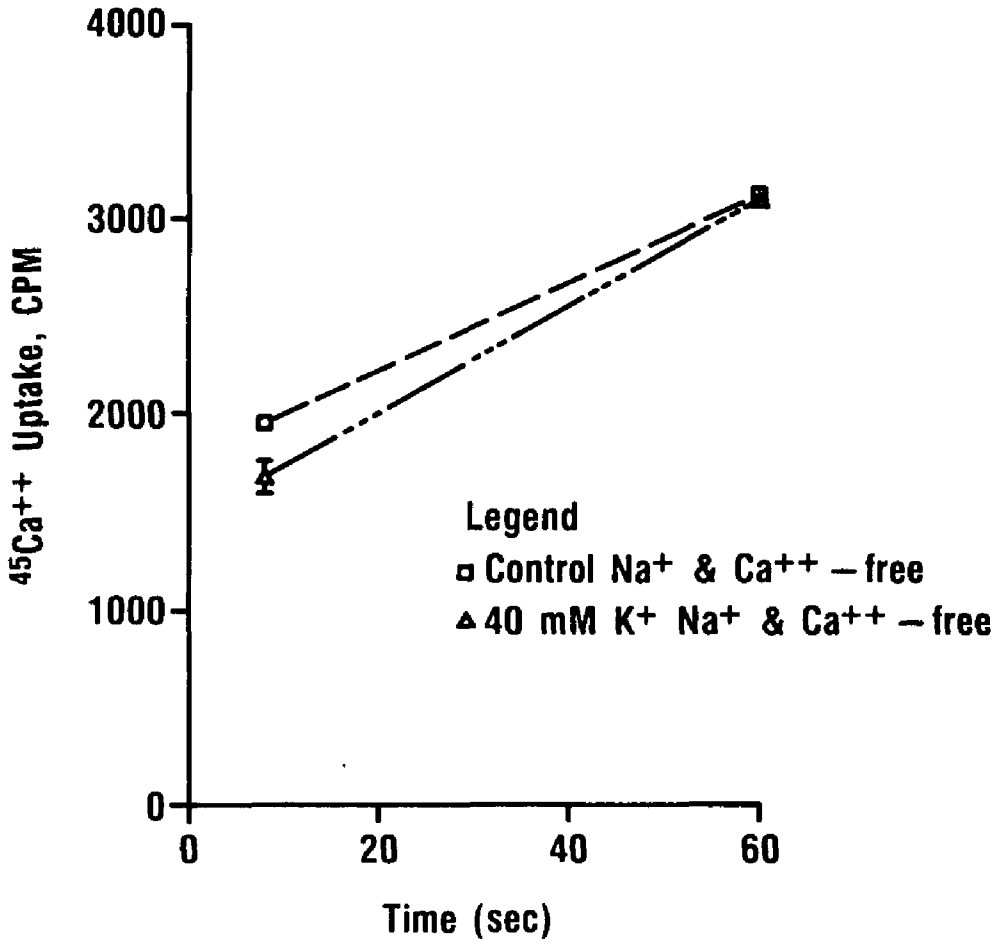


Figure 8. Uptake of $^{45}Ca^{++}$ as a function of time in Na^+free control (□), and Na^+ free depolarized (△) synaptosomes.

Table 3. Effect of Replacement of Na^+ Ions with Choline on $^{45}Ca^{++}$ Uptake by Synaptosomes in the Presence of U-50488H.

A. Time course of $^{45}Ca^{++}$ uptake in the presence of choline.

1. Uptake at rest [a].

	$^{45}Ca^{++}$ uptake CPM(+/-S.D.)
Control 8 sec	1486+/- 97
Control 1 min	3486+/-182
Control 3 min	4194+/-218
Control 5 min	4708+/-145

[a] K^+ 40 mM 8 sec control= 1531+/-52 (CPM+/-S.D.).

2. Uptake in depolarizing buffer [b].

	$^{45}Ca^{++}$ uptake CPM(+/-S.D.)
Control 8 sec	2206+/- 129
Control 30 sec	3563+/- 250
Control 1 min	4280+/- 60

[b] K^+ 5.6 mM 8 sec control= 2590+/-128 (CPM+/-S.D.).

B. Effects of U-50488H on $^{45}Ca^{++}$ uptake in the presence of choline.

1. 8 sec incubation time.

	$^{45}Ca^{++}$ uptake CPM(+/-S.D) 40mM K^{+b}	
Control	1531+/- 52	
U-50488H 10^{-4}M	1894+/- 122	*
U-50488H 10^{-5}M	1454+/- 114	
U-50488H 10-6M	1668+/- 120	*
U-50488H 10-7M	1415+/- 193	
Filter Blank	118 +/- 14	

[b] K^+ 5.6 mM control= 1486+/- 97 CPM(+/-S.D.).

* $p<0.01$ compared to 40.0 mM K^+ control. n=3.

velocity of the uptake. The uptake values for up to 30 sec are taken to represent the fast phase, and after 60 sec the late slow phase. As shown by Gripenberg et al, (1980), there is better linearity of the double reciprocal plots of the rates of calcium versus the concentration of calcium for the depolarized medium and for the fast phase. Thus, we choose the 8 sec incubation time as representative of the fast phase and designed subsequent experiments using this incubation time.

These results showed our system to be an operational system, in agreement with current publications, and that increases in uptake in depolarized synaptosomes are a reliable measure of Ca^{++} flux. We also showed that in the absence of external Na^{+} the net Na^{+}-dependent Ca^{++} uptake was inactivated as a result of depolarization. Prior depolarization of synaptosomes showed inactivation of the K^{+} stimulated calcium uptake (Table 1B). This effect has been observed by Lee & Tsien (1983).

2. EFFECTS OF THE CALCIUM CHANNEL AGONIST, BAY K 8644 ON K^{+}-STIMULATED CALCIUM UPTAKE -A significant decrease caused by BAY K 8644 was observed on calcium uptake by rat brain synaptosomes at rest and under depolarizing conditions. The uptake at 60 sec was decreased when tested with concentrations between 10^{-4} and 10^{-5} M at rest and with 10^{-5}M in high K^{+} concentration buffer (Table 4B). The uptake at 8 sec was not altered at rest or under depolarizing conditions (Table 4A).

Table 4. Effect of BAY K 8644 on Ca^{++} Uptake by Rat Brain Synaptosomes.

A. 8 sec $^{45}Ca^{++}$ incubation time.

	$^{45}Ca^{++}$ uptake CPM(+/- S.D.)	
	K^+ 5.6 mM	K^+ 40mM
Control	880 +/- 153	1325 +/- 110*
BAY K 8644 10^{-4}M	932 +/- 83	1407 +/- 61
BAY K 8644 10^{-5}M	957 +/- 268	1320 +/- 189
BAY K 8644 10^{-6}M	812 +/- 94	1320 +/- 57
BAY K 8644 10^{-7}M	829 +/- 58	1328 +/- 136
BAY K 8644 10^{-8}M	805 +/- 70	1304 +/- 117

B. 60 sec $^{45}Ca^{++}$ incubation time.

	$^{45}Ca^{++}$ uptake CPM(+/- S.D.)	
	K^+ 5.6 mM	K^+ 40 mM
Control	3084 +/- 23	6195 +/- 156 *
BAY K 8644 10^{-4}M	2377 +/- 160 *	5031 +/- 145 **
BAY K 8644 10^{-5}M	2883 +/- 103 *	5438 +/- 871
BAY K 8644 10^{-6}M	2937 +/- 163	5927 +/- 291
BAY K 8644 10^{-7}M	3105 +/- 80	5084 +/- 313
BAY K 8644 10^{-8}M	3170 +/- 38 *	5753 +/- 183
Filter Blank	164 +/- 35	138 +/- 36

* $p<0.01$ compared to K^+ 5.6 mM control.
** $p<0.01$ compared to K^+ 40 mM control.
n=3.

3. INHIBITION OF K^+-STIMULATED CALCIUM UPTAKE BY CALCIUM CHANNEL BLOCKERS- The K^+-induced increase in Ca^{++} uptake was sensitive to antagonism by verapamil at a concentration of 10^{-6}M. This Ca^{++} antagonist was effective in attenuating the depolarization-induced Ca^{++} accumulation only during the fast phase of the uptake (TABLES 5A, 5B & 6). Verapamil, a benzeneacetonitrile, is a calcium-blocker which acts at voltage sensitive calcium channels (Turner & Goldin 1985; Schramm, et al., 1986).

4. INHIBITION OF K^+-STIMULATED CALCIUM UPTAKE BY U-50488H - At rest, no antagonism of the calcium uptake was observed in the presence of U-50488H. In contrast, the K^+-induced increase in Ca^{++} uptake was antagonized by U-50488H with 8 sec incubation (Table 5A & 5B).

This decrease was concentration-related and of the same magnitude as that of verapamil and nifedipine, another calcium channel blocker (Table 6).

5. NALOXONE AND MORPHINE EFFECTS ON K^+-STIMULATED CALCIUM UPTAKE- A high concentration (10^{-5}M) of naloxone blocked calcium uptake (Table 6). Naloxone at concentrations of 10^{-7} and lower failed to decrease the Ca^{++} uptake and to block the reduction in Ca^{++} uptake induced by U-50488H. Morphine failed to significantly alter the K^+-stimulated Ca^{++} uptake at concentrations ranging from 10^{-6} to 10^{-8}M and under two different experimental conditions, in calcium-free and calcium

Table 5. Effect of Verapamil HCl and U-50488H upon Ca^{++} Uptake by Rat Brain Synaptosomes.

	$^{45}Ca^{++}$ uptake CPM(+/- S.D.)	
	K^+ 5.6 mM	K^+ 40mM
A. 8 sec $^{45}Ca^{++}$ incubation		
Control	847 +/- 72	1949 +/- 50*
Verapamil HCl 10^{-6}M	790 +/- 50	1856 +/- 59**
U-50488H 10^{-5} M	815 +/- 43	1766 +/- 66**
Filter Blank	128 +/- 24	162 +/- 29
B. 60 sec $^{45}Ca^{++}$ incubation		
Control	2512 +/- 22	4343 +/- 208*
Verapamil HCl 10^{-6}M	2509 +/- 123	4400 +/- 193
U-50488H 10^{-5} M	2423 +/- 216	4310 +/- 242
Filter Blank	138 +/- 12	148 +/- 7

* $p<0.01$ compared to 5.6 mM K^+ control.
** $p<0.01$ compared to 40 mM K^+ control.
n=3.

Table 6. Further Evaluation of the Effects of U-50488H upon Uptake by Rat Brain Synaptosomes.

A. 8 sec $^{45}Ca^{++}$ incubation

	$^{45}Ca^{++}$ uptake CPM(+/-S.D.) K^+ 40mM
Control[a]	2832 +/- 287*
U-50488H 10^{-5}M	2032 +/- 232**
U-50488H 10^{-6}M	2410 +/- 96**
U-50488H 10^{-7}M	2661 +/- 96
Verapamil HCl 10^{-5}M	1896 +/- 106**
Nifedipine 10^{-5}M	2474 +/- 76**
Naloxone HCl 10^{-5}M	2253 +/- 88**

[a] K^+ 5.6 mM control= 1215+/-123 CPM(+/-S.D.)

B. 8 sec $^{45}Ca^{++}$ incubation

	$^{45}Ca^{++}$ uptake CPM(+/-S.D.) K^+ 40mM
Control[b]	2824 +/- 99*
Naloxone HCl 10^{-7}M	2912 +/- 151
Naloxone HCl 10^{-7}M and U-50488H 10^{-6}M	2621 +/- 100**

[b] K^+ 5.6 mM control= 1452+/- 82 CPM(+/-S.D.)

* $p<0.01$ compared to 5.6 mM K^+ control. n=4.
** $p<0.01$ compared to 40 mM K^+ control. n=4.

supplemented medium, with 1 and 10 minute incubations, respectively (Table 7A & 7B).

6. EFFECTS OF EXCITATORY AMINO ACIDS ON K^+-STIMULATED CALCIUM UPTAKE IN THE PRESENCE OF Ca^{++} AND Mg^{++} IONS -Kainate (KA), and N-methyl-D-aspartate (NMDA) did not significantly alter Ca^{++} uptake by rat brain synaptosomes in a Ca^{++}-free buffer at rest or under depolarizing conditions. Likewise, the presence of Ca^{++} ions at a concentration of 2.2 mM and Mg^{++} ions at 1.3 mM were ineffective (Tables 8 and 9).

l-Glutamate markedly decreased the Ca^{++} uptake at rest and in the depolarizing buffer. The data in Table 9 demonstrate that the absence of Mg^{++} ions was not necessary for this effect on Ca^{++} uptake to be present in our system and that it was only seen with concentrations in the range of 10^{-2} to 10^{-3}M.

II. KAINIC ACID BINDING ASSAY

A. CRUDE SYNAPTIC MEMBRANE FRACTION

1. PROTEIN DETERMINATION - Approximately 0.5-0.6 mg protein/ml were used in all assays. The synaptic membrane protein levels were quantitated by the Bio-Rad method (Bradford, 1976).

2. MORPHOLOGICAL CONTROL- Examination of the synaptic membranes by electron microscopy was undertaken to determine

Table 7. Effect of Morphine on Ca^{++} Uptake by Rat Brain Synaptosomes.

A. 60 sec drug incubation, 0 Ca^{++} in preincubation medium.

	$^{45}Ca^{++}$ uptake CPM(+/- S.D.) K^+ 40mM
Control[a]	1300 +/- 68*
Morphine SO_4 10^{-6}M	1290 +/- 101
Morphine SO_4 10^{-7}M	1297 +/- 59
Morphine SO_4 10^{-8}M	1238 +/- 101

[a] K^+ 5.6 mM control= 543+/-39 CPM(+/-S.D.).

B. 10 min drug incubation, 2.2 mM Ca^{++} in preincubation medium.

	$^{45}Ca^{++}$ uptake CPM(+/-S.D.) K^+ 40mM
Control[b]	943 +/- 81*
Morphine SO_4 10^{-6}M	939 +/- 92
Morphine SO_4 10^{-7}M	930 +/- 44
Morphine SO_4 10^{-8}M	852 +/- 74

[b] K^+ 5.6 mM control= 508+/-44 CPM(+/-S.D.).

* $p<0.01$ compared to 5.6 mM K^+ control. n=3.

Table 8. Effect of Excitatory Amino Acids on Ca^{++} Uptake by Rat Brain Synaptosomes.

	$^{45}Ca^{++}$ uptake CPM(+/- S.D.) K^+ 5.6 mM
Control[a]	1747 +/- 118
KA 10^{-4}M	1625 +/- 26
KA 10^{-5}M	1659 +/- 48
KA 10^{-6}M	1641 +/- 116
KA 10^{-7}M	1636 +/- 103
KA 10^{-8}M	1658 +/- 100
Filter Blank	76 +/- 10

[a] K^+ 40 mM control=3164+/-252 CPM(+/-S.D.).

	$^{45}Ca^{++}$ uptake CPM(+/- S.D.) K^+ 5.6 mM
Control	1443 +/- 49
NMDA 10^{-5}M	1694 +/- 144
NMDA 10^{-6}M	1729 +/- 91
NMDA 10^{-7}M	1721 +/- 111
Filter Blank	192 +/- 64

	$^{45}Ca^{++}$ uptake CPM(+/- S.D.)	
	K^+ 5.6 mM	K^+ 40 mM
Control	3036 +/- 166	7126 +/- 107*
L-Glutamate 10^{-2}M	773 +/- 90*	701 +/- 20**
L-Glutamate 10^{-3}M	2984 +/- 340	5654 +/- 550**
Filter Blank	192 +/- 64	269 +/- 80

* $p<0.05$ compared to 5.6 mM K^+ control. n=4.
** $p<0.05$ compared to 40 mM K^+ control. n=4.

Table 9. Effect of Excitatory Amino Acids and Mg^{++} ions on Ca^{++} Uptake by Rat Brain Synaptosomes.

A. Effect of Mg^{++} ions upon $^{45}Ca^{++}$ uptake

	$^{45}Ca^{++}$ uptake CPM(+/-S.D.)	
	K^+ 5.6 mM	K^+ 40mM
Control	1443 +/- 49	1848 +/- 109*
Mg^{++} 1.3 mM	1143 +/- 49	1637 +/- 84**
Filter Blank	192 +/- 64	269 +/- 80

B. $^{45}Ca^{++}$ uptake in Mg^{++}-free buffer.

	$^{45}Ca^{++}$ uptake CPM(+/-S.D.)	
	K^+ 5.6 mM	K^+ 40mM
Control	1443 +/- 49	1848 +/- 109*
KA 10^{-3}M	674 +/- 13*	1446 +/- 31
KA 10^{-4}M	1375 +/- 67	1900 +/- 149
KA 10^{-5}M	1261 +/- 81*	2316 +/- 57
KA 10^{-6}M	1237 +/- 34	2026 +/- 72
Filter Blank	192 +/- 64	269 +/- 80
Control	1443 +/- 49	1848 +/- 109*
l-Glutamate 10^{-3}M	410 +/- 4*	558 +/- 21
l-Glutamate 10^{-4}M	1078 +/- 47	1774 +/- 196
l-Glutamate 10^{-5}M	1188 +/- 12	1934 +/- 109
l-Glutamate 10^{-6}M	1223 +/- 21	1652 +/- 153
Filter Blank	192 +/- 64	269 +/- 80
Control	1443 +/- 49	1848 +/- 109*
NMDA 10^{-3}M	598 +/- 26*	1265 +/- 63
NMDA 10^{-4}M	1312 +/- 57	1826 +/- 69
NMDA 10^{-5}M	1227 +/- 38	2023 +/- 62
NMDA 10^{-6}M	1138 +/- 52	1894 +/- 99
Filter Blank	192 +/- 64	269 +/- 80

* $p<0.05$ compared to 5.6 mM K^+ control. n=4.
** $p<0.01$ compared to 40 mM K^+ control. n=4.

the homogeneity of the cellular material collected. The identifiable subcellular components included myelin and unidentified membranous material (Figure 9).

B. KAINIC ACID BINDING ASSAY TECHNIQUE

1. SATURABILITY - The bound radioligand was plotted against the free radioligand at concentrations ranging from 1.5 to 200 nM. The specific binding data were obtained by using data expressed in CPM. Total specific binding of (^{3}H)KA was defined as the difference between the total binding with radioligand alone and nonspecific binding measured in the presence of 0.1 mM unlabeled kainic acid. Total binding and non-specific binding curves increased linearly with increasing concentrations of the radioligand (Figure 10).

a. BINDING IN Ca^{++}-FREE BUFFER - The specific binding of (^{3}H)KA to forebrain synaptic membranes was saturable with a receptor density maximum (B_{max}) of 1.28 +/- 0.008 pmol/mg protein, and a dissociation constant (K_d) of 32.93 +/- 2.8 nM in the Ca^{++}-free buffer. To confirm saturability, a double reciprocal plot of 1/bound KA versus 1/free KA was constructed. Linear regression analysis of these points yielded a y-intercept and a x-intercept supporting the saturability of this system (Table 10 & Figure 11).

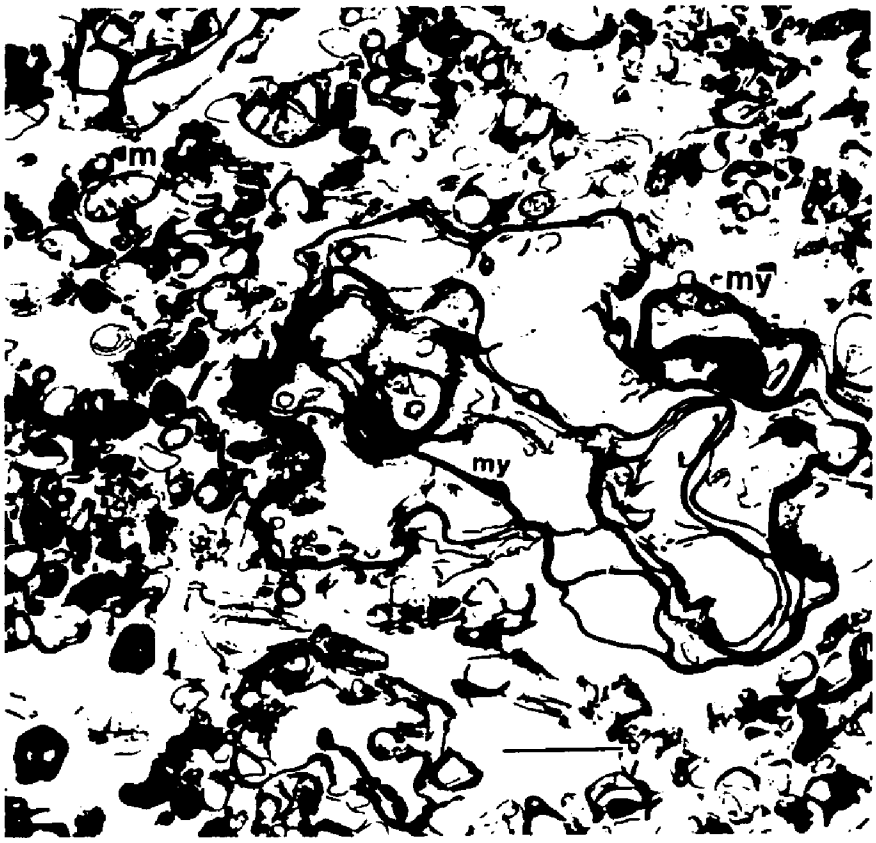


Figure 9. Electron micrograph of mouse synaptic membranes. Morphologic control. Myelin (my) 65,000x.

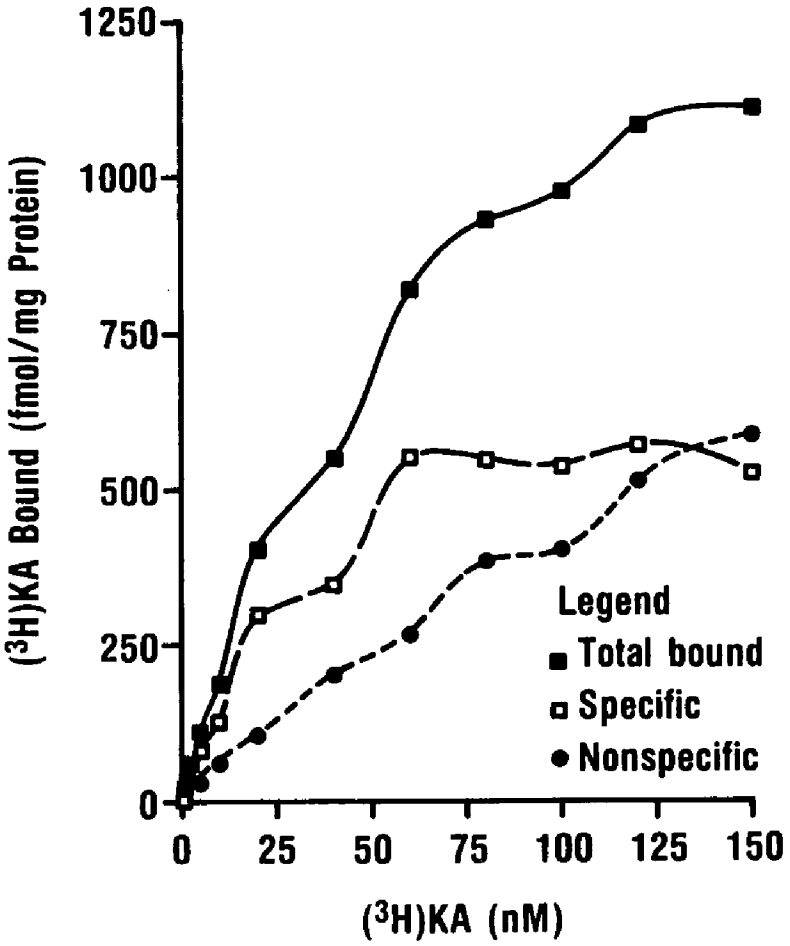


Figure 10. Saturation of (^{3}H)KA binding to rat forebrain synaptic membranes in Ca^{++}-free buffer as a function of radioligand concentration. (■) Total Binding; (□) Specific Binding; (●) Non-Specific Binding.

Table 10. Kainic Acid Binding Assay. Double Reciprocal Plot Data

	Intercept	
	Y	X
A. Tris/Acetate buffer		
Ca^{++} free	1.050	-0.076
Ca^{++} 1.25 mM added	0.398	-0.024
Ca^{++} 1.25 mM and U-50488H 10^{-5} M	0.538	-0.037
Ca^{++} 2.5 mM added	0.676	-0.045
B. Cl^{-} containing buffer		
Ca^{++} free	2.390	-0.016
Ca^{++} 1.25 mM added	1.980	-0.049
Ca^{++} 2.5 mM added	2.820	-0.182
Ca^{++} 2.5 mM and U-50488H 10^{-5} M	1.646	-0.084

b. BINDING IN THE PRESENCE OF Cl^- IONS- The specific binding of (^{3}H)KA to forebrain synaptic membranes was saturable with a receptor density maximum (B_{max}) of 1.30 +/- 0.01 pmol/mg protein, and a dissociation constant (K_d) of 22.32 +/- 1.0 nM in the Tris/HCl buffer. To confirm saturability, a double reciprocal plot of 1/bound KA versus 1/free KA was constructed. Linear regression analysis of these points yielded a y-intercept and a x-intercept supporting the saturability of this system (Table 10).

c. BINDING IN THE PRESENCE OF Ca^{++} IONS- The specific binding of (^{3}H)KA to forebrain synaptic membranes in a Tris/Acetate buffer was saturable with a receptor density maximum (B_{max}) of 1.76 +/- 0.1 pmol/mg protein, and a dissociation constant (K_d) of 33.72 +/- 2.0 nM in the presence of 1.25 mM Ca^{++}. To confirm saturability, a double reciprocal plot of 1/bound KA versus 1/free KA was constructed. Linear regression analysis of these points yielded a y-intercept and a x-intercept supporting the theory of the saturability of this system (Table 10). In the presence of 2.5 mM Ca^{++}, the specific binding of (^{3}H)KA to forebrain synaptic membranes was saturable with a receptor density maximum (B_{max}) of 1.80 +/- 0.1 pmol/mg protein, and a dissociation constant (K_d) of 61.24 +/- 3.0 nM in the 2.5 mM Ca^{++} buffer. To confirm saturability, a double reciprocal plot of 1/bound KA versus 1/free KA was constructed. Linear regression analysis of these points yielded a y-intercept and a x-intercept supporting the theory

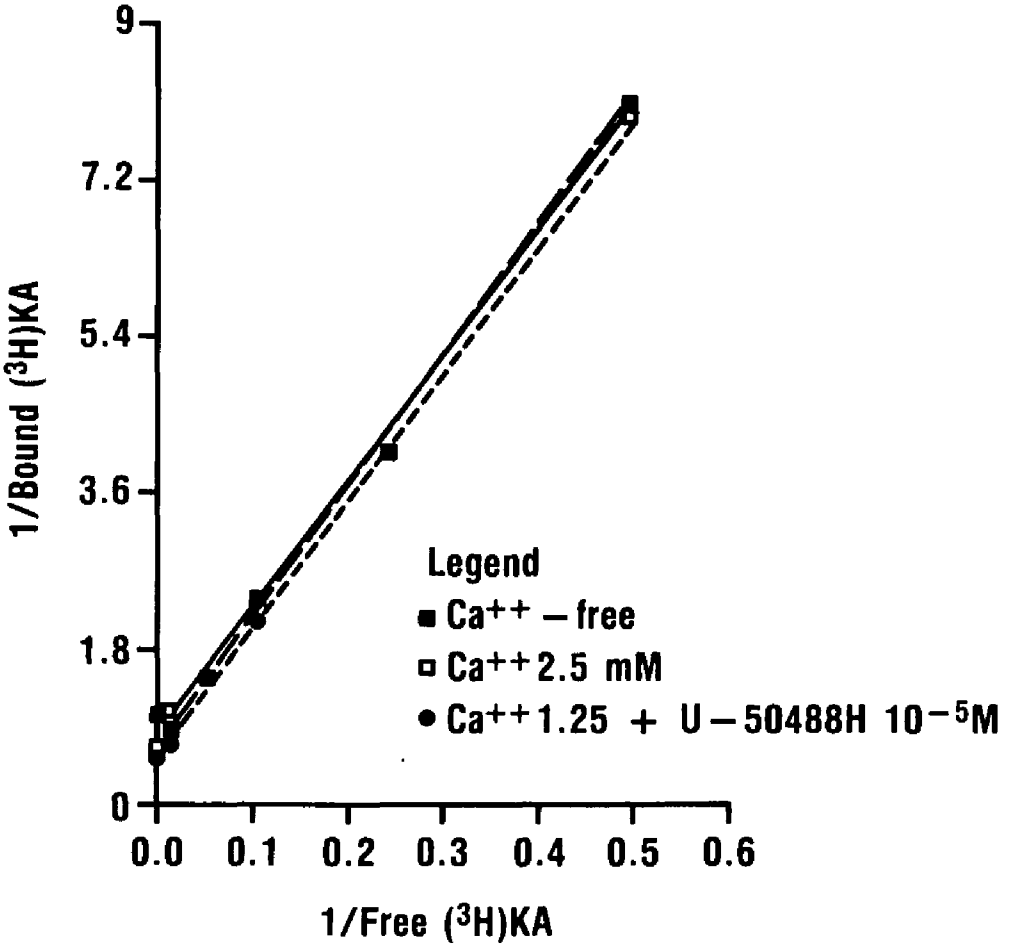


Figure 11. Double reciprocal plot of (^{3}H)KA binding to mouse forebrain synaptic membranes in a Ca++-free buffer.

Table 11. Kainic Acid Binding Parameters for Mouse Forebrain Membranes.

A. Tris/Acetate buffer 50 mM, pH=7.1.

1. Ca^{++} free buffer n=4

Parameter	B_{max} (pM/mg protein)	K_d (nM)
Weighted average	1.28 +/- 0.01	32.93 +/-3
Intra Assay % CV	23.8	39.9
Inter Assay % CV	6.4	42.7

2. Ca^{++} added

a. 1.25 mM Ca^{++} n=2

Weighted Average	1.76 +/- 0.10	33.72 +/-2
Intra Assay % CV	8.1	9.8
Inter Assay % CV	8.5	49.0

b. 2.50 mM Ca^{++} n=3

Weighted Average	1.80 +/- 0.10	61.22 +/-3
Intra Assay % CV	14.1	9.5
Inter Assay % CV	21.0	32.5

B. Tris/HCl buffer 50 mM, pH=7.1.

1. Cl- ions buffer n=4

Parameter	B_{max} (pM/mg protein)	K_d (nM)
Weighted average	1.30 +/- 0.01	22.32 +/-1
Intra Assay % CV	12.2	13.3
Inter Assay % CV	21.5	49.9

2. Ca^{++} added

a. 1.25 mM Ca^{++} n=1

Weighted Average	2.68 +/- 1.42	127.40 +/- 81

b. 2.50 mM Ca^{++} n=2

Weighted Average	1.59 +/- 0.20	105.0 +/-4
Intra Assay % CV	30.1	5.8
Inter Assay % CV	42.4	82.6

of the saturability of this system (Figure 11 & Tables 10 & 11).

d. BINDING IN THE PRESENCE OF Ca^{++} AND Cl^{-} IONS- The specific binding of (^{3}H)KA to forebrain synaptic membranes was saturable with a receptor density maximum (B_{max}) of 2.68 +/- 1.42, and a dissociation constant (K_d) of 127.40+/-81 in the 1.25 mM Ca^{++} buffer. To confirm saturability, a double reciprocal plot of 1/bound KA versus 1/free KA was constructed. Linear regression analysis of these points yielded a y-intercept and a x-intercept supporting the theory of the saturability of this system (Tables 10 & 11 & Figure 12).

In the presence of 2.5 mM Ca^{++}, the specific binding of (^{3}H)KA to forebrain synaptic membranes was saturable with a receptor density maximum (B_{max}) of 1.59 +/- 0.17, and a dissociation constant (K_d) of 104.63+/-81 in the 2.50 mM Ca^{++} buffer. To confirm saturability, a double reciprocal plot of 1/bound KA versus 1/free KA was constructed. Linear regression analysis of these points yielded a y-intercept and a x-intercept supporting the theory of the saturability of this system (Tables 10 & 11).

2. SPECIFICITY OF BINDING

a. NON-LABELEDED KAINATE AND 1-GLUTAMATE- Unlabeled kainic and 1-glutamic acid effects on the binding of radiolabeled kainic

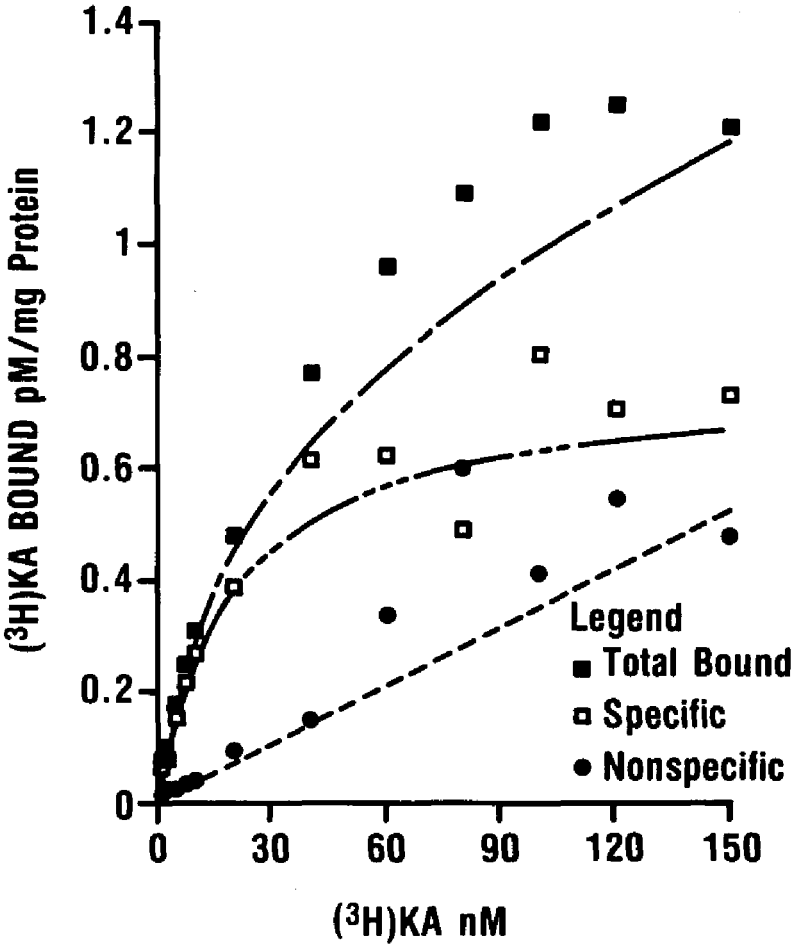


Figure 12. Saturation of $^{(3}$H)KA binding to rat forebrain synaptic membranes in the presence of Cl^- and Ca^{++} ions as a function of radioligand concentration. (■) total binding; (□) specific binding; (●) non-specific binding.

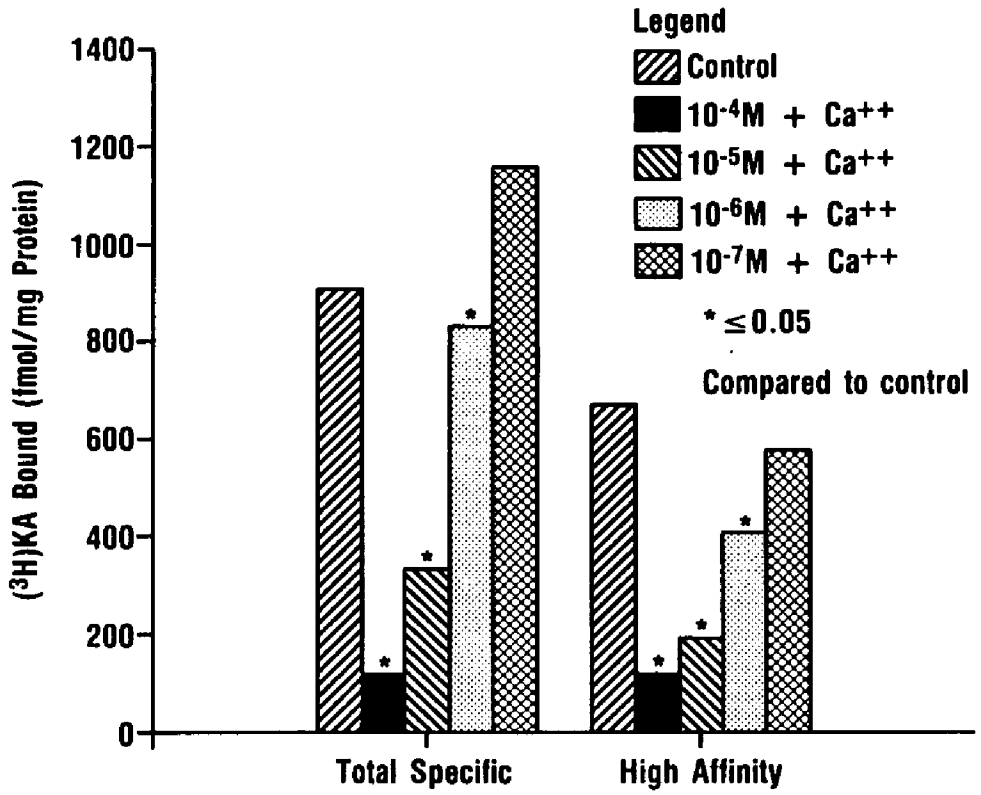


Figure 13. Effect of l-glutamic acid on (^{3}H)KA binding to mouse forebrain synaptic membranes.

acid resulted in a competitive inhibition effect in the presence of Ca^{++} and Cl^{-} ions. The mean concentration of l-glutamate that inhibited the binding of the radioligand by 50% (IC_{50}) was $8.4x10^{-6}$ M for the total specific binding, and $1.9x10^{-7}$ M for the high affinity binding (Figure 13).

b. U-50488H- The effect of U-50488H on the KA binding to synaptic membranes is shown in Figure 14B. The mean IC_{50} for U-50488H was $2.6x10^{-5}$ M for the total specific binding, and $4.9x10^{-5}$ M for the high affinity binding. U-50488H did not compete with the radioligand for binding on the rat forebrain synaptic membranes in a preparation without Ca^{++} (Figure 14A).

3. SCATCHARD ANALYSIS - The B_{max} and the K_d were determined from the Scatchard analysis plot. The radioactivity measured in the samples containing cold KA was considered nonspecific binding (NSB) and was subtracted from all samples to obtain specific binding for each concentration. The maximum binding, or B_{max} was measured in the absence of cold KA. Plots of the ratio of bound to free radioligand on the y-axis and the concentration of bound radioligand (nM) on the x-axis were constructed for each different ionic buffer (Figures 15 & 16). The resulting curves were linear, with a negative slope. The total number of receptor sites (B_{max}) was obtained by linear regression. The constants of dissociation were obtained by taking the negative inverse of the slopes of the lines. Results are summarized in Tables 11, 12 and the statistical evaluations listed in Tables 14 and 15. The correlation

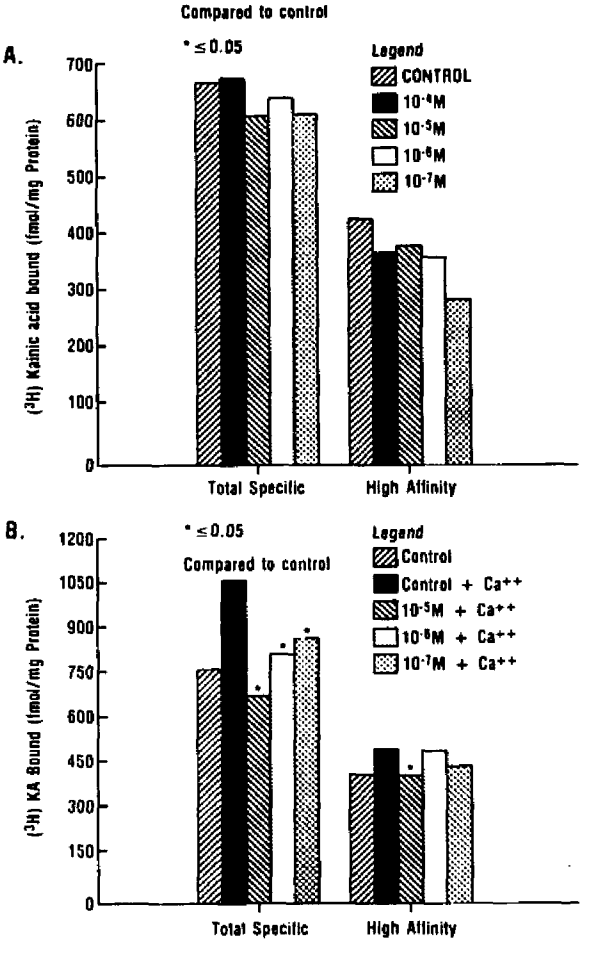


Figure 14. Effect of U-50488H on (^{3}H)KA binding to mouse forebrain synaptic membranes.

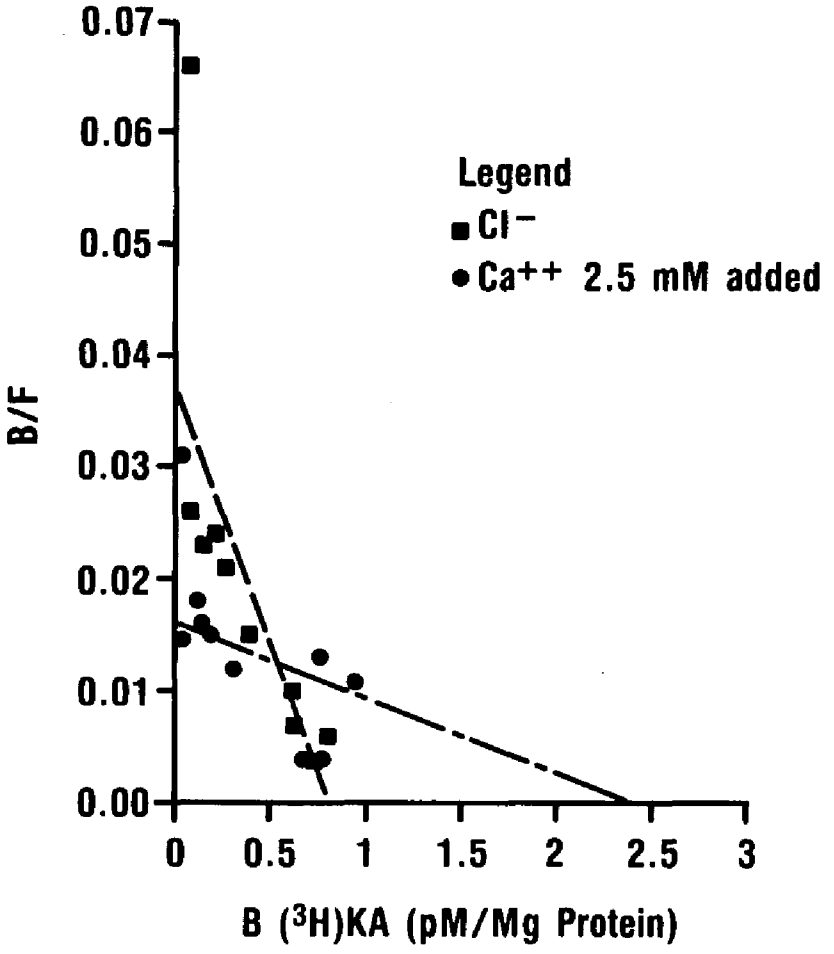


Figure 15. Scatchard plot of specific (^{3}H)KA binding to mouse forebrain synaptic membranes in a Tris/HCl buffer.

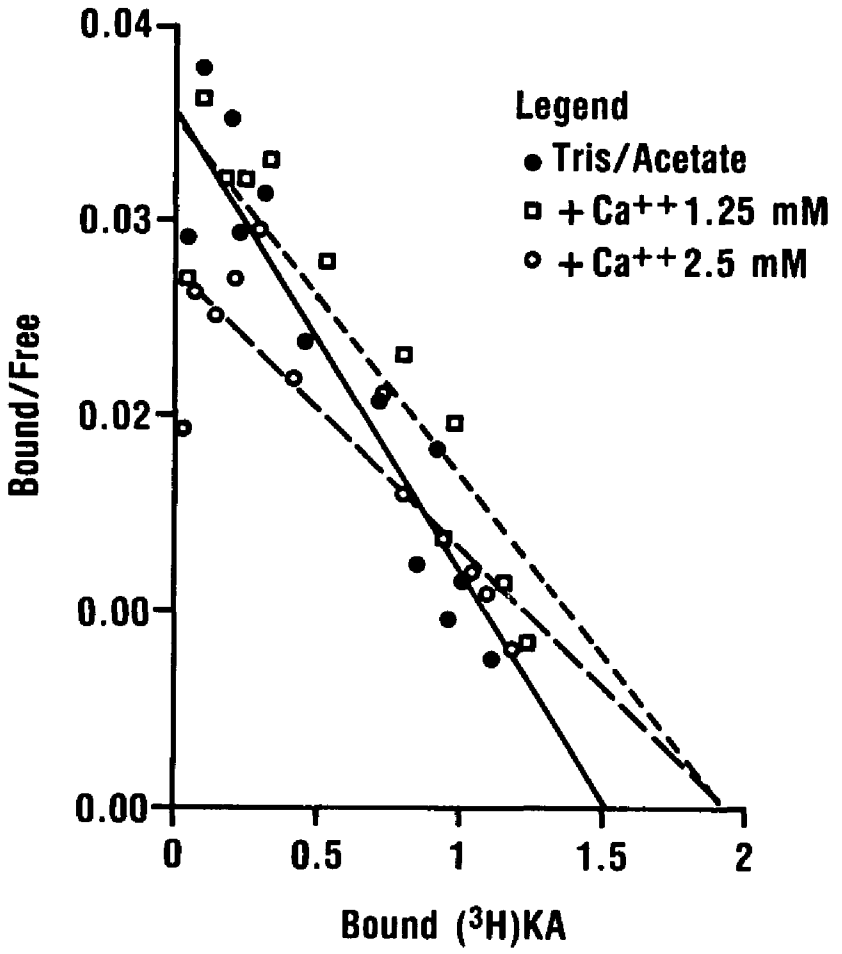


Figure 16. Scatchard plot of specific (^{3}H)KA binding to mouse forebrain synaptic membranes in a Ca^{++}-free buffer and in the presence of Ca^{++} ions.

Table 12. Kainic Acid Binding Parameters for Mouse Forebrain membranes in the Presence of U-50488H 10^{-5}M. Scatchard Analysis.

Assay #	B_{max} (pM/mg protein)	K_d (nM)	r
I. Tris/Acetate buffer.			
A. Ca^{++} 1.25 mM			
	1.79 +/- 0.08	26.35 +/- 2	0.9451
B. Ca^{++} 2.50 mM			
	1.93 +/- 0.09	33.60 +/- 5	0.3245
II. Tris/HCl buffer.			
A. Ca^{++} 2.50 mM added			
	1.17 +/- 0.25	28.47 +/- 9	0.4822

coefficients for kainic acid binding parameters by this analysis are summarized in Table 13.

a. INTER- AND INTRA- ASSAY COEFFICIENTS OF VARIATION FOR (^{3}H)KA RECEPTOR BINDING TO MOUSE FOREBRAIN SYNAPTIC MEMBRANES -Inter-assay and intra-assay percent coefficients of variation (CV) and the combined mean +/- SEM B_{max} for each ionic condition are shown in (Tables 10 & 12).

4. NON-LINEAR ANALYSIS - The Bmax and the K_d were determined by non-linear analysis regression using a software Receptor Binding Program Package (Metzer & Weiner, 1984). The radioactivity measured in the samples containing cold KA was considered nonspecific binding (NSB) and was substracted from all samples to obtain specific binding for each concentration. The maximum binding or B_{max} was measured in the absence of cold KA. Plots of the ratio of bound to free radioligand on the y-axis and the concentration of bound radioligand (nM) on the x-axis were constructed for each different ionic buffer. The resulting curves were linear in nature with an negative slope. By non-linear regression to its intersecting point on the abscissa the total number of receptor sites (B_{max}) was obtained. The constant of dissociation (K_d) was obtained by taking the negative inverse of the slopes of the lines.

Efforts to discriminate the low and high affinity sites

Table 13. Correlation Coefficients for Kainic Acid Binding Parameters by Scatchard Analysis.

Comparison	r obs	Prob(r>|r|/Ho:rho=0)[a]
I. Ca^{++} free buffer.		
B_{max} vs. K_d	0.1922	-0.8078
II. Tris/Acetate buffer + Ca^{++} 2.50 mM.		
B_{max} vs. K_d	0.0149	-0.9997
III. Cl- and Ca^{++} 2.50 mM.		
B_{max} vs. K_d	ND	1.0000

[a] Probability of an r greater than the absolute value of the observed r given the null hypothesis of no correlation.

Table 14. Statistical Significance of Kainic Acid Binding Parameters by Scatchard Analysis.

Comparison			
X1	vs.	X2	p
B_{max} (pmol/mg protein)			
Control	vs.	mM Ca^{++} added	
Ca^{++} free	vs.	1.25	0.0972
Ca^{++} free	vs.	2.50	0.0650
Cl^- added	vs.	1.25	0.4166
Cl^- added	vs.	2.50	0.5032
K_d (nM)			
Control	vs.	mM Ca^{++} added	
Ca^{++} free	vs.	1.25	0.7788
Ca^{++} free	vs.	2.50	0.0000 *
Cl^- added	vs.	1.25	0.3428
Cl^- added	vs.	2.50	0.0112 *

* X1 different from X2 ($p<0.05$).

Table 15. Statistical Significance of Kainic Acid Binding Parameters by Scatchard Analysis in the Presence of U-50488H (10^{-5}M).

Comparison			
X1	vs.	X2	p
B_{max} (pmol/mg protein)			
Control vs.		U-50488H treated	
Ca^{++} free + Ca^{++} 1.25 mM		10^{-5} M	0.7770
Ca^{++} free + Ca^{++} 2.50 mM		10^{-5} M	0.4989
Cl- + Ca^{++} 2.50 2.50 mM		10^{-5} M	0.3036
K_d (nM)			
Control vs.		U-50488H treated	
Ca^{++} free + Ca^{++} 1.25 mM		10^{-5} M	0.0272 *
Ca^{++} free + Ca^{++} 2.50 mM		10^{-5} M	0.0000 *
Cl- + Ca^{++} 2.50 mM			0.4456

* X1 different from X2 ($p<0.05$).

statistically were unsuccessful by this method. A very high standard error was necessary for the two site model to fit the data, and no reduction of the sum of squares residuals was obtained. The presence of two sites has been shown by London & Coyle (1979) and Simon, et al. (1976). The fact that we can not statistically demonstrate their presence does not invalidate their biological significance and our results are expresed as B_{max} and K_d corresponding to the horizontal portion of the curvilinear lines. These values of K_d correspond to the low affinity component of the curves.

5. DISPLACEMENT OF (^{3}H)KA FROM HIGH AND LOW AFFINITY BINDING SITES-

a. TOTAL SPECIFIC BINDING SITES- l-Glutamate displaced the total specific binding of (^{3}H)KA from the receptor sites of the mouse forebrain in a dose-dependent manner in the Ca^{++}-free medium and in the presence of Ca^{++} and Cl^- ions (Figure 13).

U-50488H did not significantly alter the specific binding of (^{3}H)KA to the receptor sites in the mouse forebrain homogenate in the Ca^{++}-free buffer. U-50488H did decrease the binding to the receptor sites in a dose-related manner in the presence of Ca^{++} ions (Figure 14B).

b. HIGH AFFINITY BINDING SITES- Advantage was taken of the fact that the radioligand dissociates slowly ($T_{1/2}$= 90 min) from high affinity sites but nearly instantaneously from low affinity sites. The difference between total specific binding and high affinity binding represents the low affinity binding sites.

l-Glutamate displaced the (^{3}H)KA binding to the high affinity binding sites in a dose-dependent manner in the Ca^{++}-free medium, and

in the presence of Ca^{++} and Cl^{-} ions (Figure 13). U-50488H did not significantly alter the binding of (^{3}H)KA to the high affinity sites in the Ca^{++}-free buffer. U-50488H did decrease the binding to the high affinity sites in a dose-dependent manner in the presence of Ca^{++} ions (Figure 14B).

c. STATISTICAL ANALYSIS- Student's t-test was used to statistically analyze the data. No significant change was observed in B_{max}. The K_d values in the presence of Ca^{++} were significantly increased at both concentrations of Ca^{++} used. The results are shown in Table 15.

6. RADIOLIGAND DEGRADATION - Degradation of the labeled (^{3}H)KA is not expected to exceed 1% per month in the first six months after the initial analysis, but thereafter may accelerate. The radioligand was used as soon as it was shipped and stored at 2 C as advised by the manufacturer [r].

III 2-(^{3}H)-DG UPTAKE IN VARIOUS MOUSE BRAIN REGIONS

A. BRAIN DISSECTION- The dissection of the septum, corpus striatum and hippocampus was done according to the diagram shown in Figure 17, following Montemurro & Dukelow (1972).

B. 2-(^{3}H)-DG UPTAKE ASSAY -There were no significant differences between the saline control and the U-50488H treated group for the uptake of 2-deoxyglucose (2-DG) in the septum, corpus striatum, and hippocampus (unpaired t-test).

The data in Table 16 indicate that the i.v. administration of kainic acid at 30 mg/Kg produced an increase of 2-DG uptake of 223, and 209% in hippocampus, and septum respectively, when compared with saline controls (Footnotes [a] and [b]). No significant changes were seen in the striatum.

U-50488H by itself or in the presence of KA did not significantly alter the uptake of 2-DG (Table 16) in the brain tissues studied nor did it block the increases induced by kainic acid.

Diazepam at 10 mg/Kg decreased 2-(^{3}H)-DG uptake to 67% of the saline control values in hippocampus. In the presence of 30 mg/Kg kainic acid, diazepam at a dose of 10 mg/Kg decreased the 2-DG uptake to 40%, 37% and 37% of the kainic acid control values in mouse hippocampus, corpus striatum and septum, respectively. Diazepam at doses of 3 and 1 mg/Kg did not produce these marked effects. Phenytoin (100 mg/Kg) decreased 2-(^{3}H)-DG uptake to 72% and 74% of the saline values in hippocampus and corpus striatus, respectively.

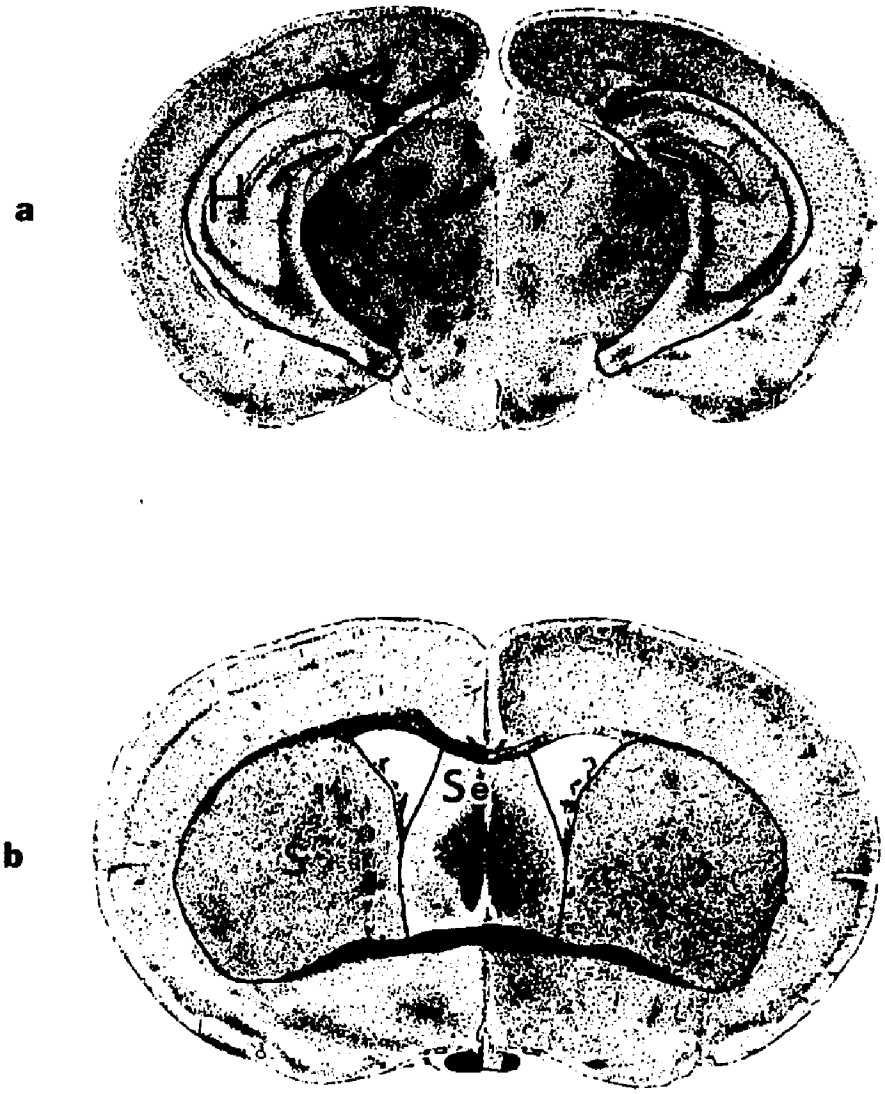


Figure 17. Drawing of mouse brain regions dissected for (^{3}H)DG uptake. S= C. Striatum; H= Hippocampus; Se= Septum.

Table 16. Effects of U-50488H on 2-(^{3}H)DG Uptake in Mouse Brain

GROUP	DOSE mg/Kg	PERCENT OF CONTROL (CPM+/-S.D.) [a] HIPPOCAMPUS	C. STRIATUM	SEPTUM
Saline		100	100	100
U-50488H	100	117	81	87
Diazepam	10	67*	89	70
Phenytoin	100	72*	74*	87
	30	144*	94	95

GROUP	DOSE mg/Kg	PERCENT OF CONTROL (CPM+/-S.D.) [a] HIPPOCAMPUS	C. STRIATUM	SEPTUM
KA iv	30	100	100	100
U-50488H + KA iv	100 30	126	96	132
U-50488H + KA iv	30 30	87	103	87
Diazepam + KA iv	10 30	40*	37*	37*
	3	111	96	78
	1	85	72*	83
Phenytoin + KA iv	30 30	73*	77*	63
	10	107	89	75

* $p<0.05$, Student's t-test. df=15

[a] Saline control CPM/mg of tissue(+/-S.D.):

HIPPOCAMPUS	C. STRIATUM	SEPTUM
183+/-24	263 +/- 54	187 +/- 22

[b] KA Control CPM/mg of tissue(+/-S.D.):

HIPPOCAMPUS	C. STRIATUM	SEPTUM
408+/-132	252 +/- 73	392 +/- 98

A 30 mg/Kg dose significantly blocked the increase induced by kainic acid (Table 16).

IV IMAGE ANALYSIS QUANTITATION OF KAINIC ACID LESIONS IN MOUSE HIPPOCAMPUS AND THE PROTECTIVE EFFECTS OF THE ADMINISTRATION OF U-50488H.

A. HISTOPATHOLOGY

1. DESCRIPTION OF KA LESIONS - Histopathological changes with KA were restricted to the neurons and associated neuropil of the granular layer in the CA3 region of the hippocampus. Extensive degeneration and necrosis of neurons resulted in dark, shrunken cell bodies and vacuolization of the neuropil associated with pyknotic neurons. The site of injection was confirmed histologycally, a typical injection site is shown in Figure 18.

2. PHOTOGRAPHIC DISPLAY- Typical KA lesions in the CA3 region of the hippocampus are shown in Figure 4 at the five levels used for quantitation in this study.

The effect of U-50488H administration on kainic acid-induced lesions is shown in Figure 5 and Table 17. The lesions were decreased in size after treatment with U-50488H (100 mg/Kg). Less vacuolization and degeneration of neurons was present in the CA3 region of the hippocampus

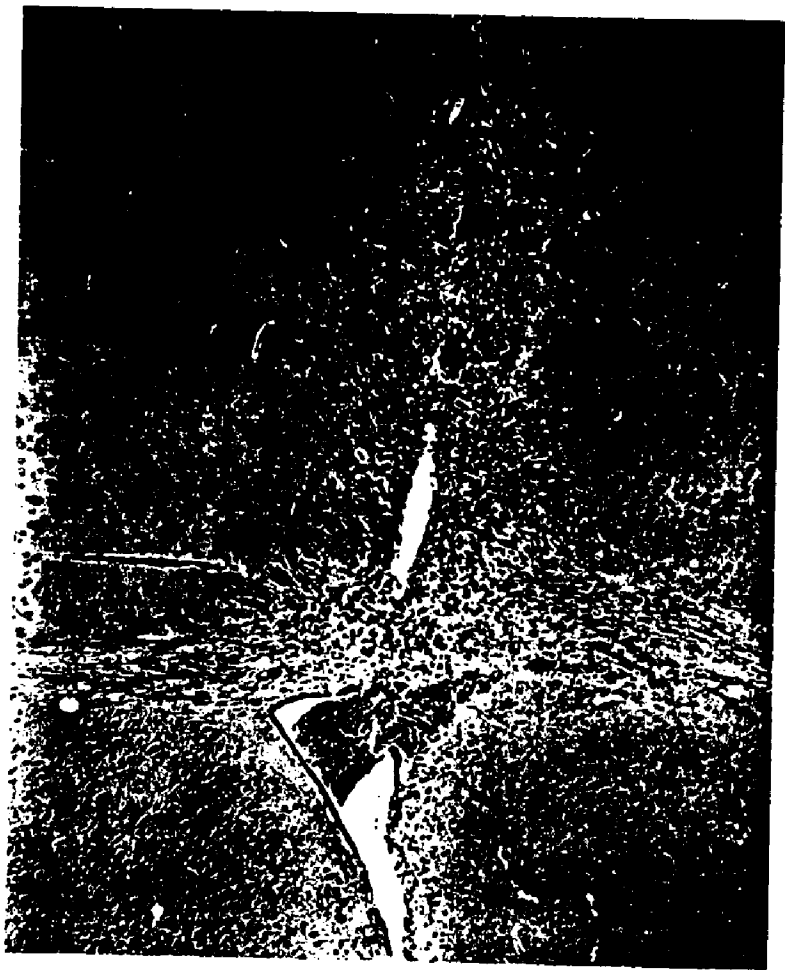


Figure 18. Site of KA intracerebroventricular injection. H and E stain. 20X.

after treatment, but patches of necrotic changes were still observed.

B. IMAGE ANALYSIS

1. ACQUISITION AND PROCESSING OF IMAGES - Images of a 200x200 pixel area from the CA3 region of the hippocampus of every animal in every group were acquired using a Nikon Magiscan Image Analysis system incorporating a Dage black and white video camera mounted on a Nikon Fluophot microscope fitted with a 20x objective lens. All operations of the image processor were controlled by the computer. The computer digitized the signal to 6-byte resolution and stored it for image processing. A representative KA treated group is shown in Figure 19.

2. QUANTITATION - Total area of vacuolization (mm^2) was measured for each animal on both sides of the brain. The data are shown in Table 17. In the injection side, the area of vacuolization (mm^2) was 1.45 +/- 0.90 and 0.22 +/- 0.43 for KA and KA+ U-50488H respectively. The contralateral side area of vacuolization (mm^2) was 0.37 +/- 0.63 and 0.01 +/- 0.02 for KA and combined treatment respectively.

3. STATISTICAL ANALYSIS - The data were analyzed by one-way analysis of variance by pairwise comparison of treatment means using Fisher's Least Significant Difference (LSD) (Table 18).

Table 17. Effects of U-50488H on the Area of KA Induced Vacuolization in the CA3 Region of the Mouse Hippocampus.

		Total Area of Vacuolization (mm^2)	
TREATMENT	N	SIDE 1[a]	SIDE 2[b]
Saline	8	0.05 +/- 0.06	0.01 +/- 0.03
KA 0.08 ug icv	5	1.45 +/- 0.90 *	0.37 +/- 0.63
U-50488H 100mg/Kg	5	0.00 +/- 0.00	0.00 +/- 0.00
KA 0.08 ug icv + U-50488H 100 mg/Kg	5	0.22 +/- 0.43 **	0.01 +/- 0.02**

[a] Injection side.
[b] Contralateral side.
* statistically significant when compared with saline control ($p<0.05$).
** statistically significant when compared with KA control ($p<0.05$).

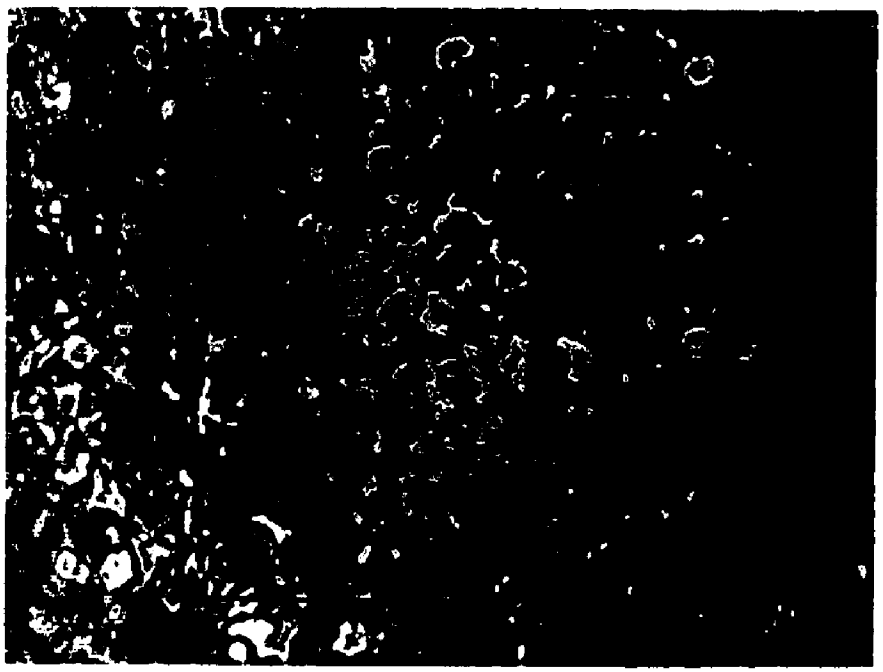


Figure 19. Digitized image of mouse hippocampus lesion with KA.

For the LSD analysis, those comparisons which were applied to test the null hypotheses that all of the populations are identical against the alternative that some of the populations tend to furnish greater observed values than other populations. This test also confirmed the significant difference between the KA treated group and the combined treatment with U-50488H + KA. For the Kruskal-Wallis test (chi-square approximation) a PROB > CHISQ = 0.0002 was used in the contralateral side and a PROB > CHISQ = 0.0001 in the injection side. The significance of the test for all comparisons is shown in Tables 19 & 20.

Table 18. Analysis for Variable Mean Area of Vacuolization Induced by KA in the CA3 Region of the Mouse Hippocampus.

Wilcoxon Scores (Rank Sums)

Side 2 (Contralateral Side)

GROUP	N	SUM OF SCORES	EXPECTED UNDER HO	STD DEV UNDER HO	MEAN SCORE
Saline	6	122.50	117.00	24.35	20.42
KA	7	212.00	136.50	25.89	30.29
U-50488H	6	61.00	117.00	24.35	10.17
KA + U-50488H	5	98.00	117.00	24.35	16.33

Kruskal-Wallis test (chi-square approximation)
CHISQ= 24.62 df = 5 PROB > CHISQ= 0.0002

Side 1 (Injection Side)

GROUP	N SCORES	SUM OF SCORES	EXPECTED UNDER HO	STD DEV UNDER HO	MEAN SCORE
Saline	6	114.00	117.00	24.86	19.00
KA	7	245.00	136.50	26.43	35.00
U-50488H	6	61.50	117.00	24.86	10.25
KA + U-50488H	5	123.50	117.00	24.86	20.58

Kruskal-Wallis test (chi-square approximation)
CHISQ= 30.37 df = 5 PROB > CHISQ= 0.0001

Table 19. Analysis for Variable Mean Area of Vacuolization Induced by KA in the CA3 Region of the Mouse Hippocampus.

Kruskal-Wallis Test (Chi-Square Approximation)

Side 1 (Contralateral Side)
Treatment Comparison

		N	DF	CHISQ	PROB > CHISQ
Saline vs.	KA	7	1	3.45	0.0633
	U-50488H	6	1	5.40	0.0201
	KA + U-50488H	6	1	0.33	0.5683

Side 2 (Injection Side)
Treatment Comparison

		N	DF	CHISQ	PROB > CHISQ
Saline vs	KA	7	1	9.00	0.0027
	U-50488H	6	1	6.66	0.0099
	KA + U-50488H	6	1	0.41	0.5218

Side 1 (Contralateral Side)
Treatment Comparison

		N	DF	CHISQ	PROB > CHISQ
KA vs.	Saline	1	1	3.45	0.0633
	U-50488H	6	1	9.25	0.0023
	KA + U-50488H	6	1	4.64	0.0312

Side 2 (Injection Side)
Treatment Comparison

		N	DF	CHISQ	PROB > CHISQ
KA vs.	Saline	6	1	9.00	0.0027
	U-50488H	6	1	9.10	0.0026
	KA + U-50488H	6	1	9.00	0.0027

Table 20. Analysis for Variable Mean Area of Vacuolization Induced by KA in the CA3 Region of the Mouse Hippocampus.

WILCOXON 2-SAMPLE TEST (NORMAL APPROXIMATION)

Side 1 (Contralateral Side)

Treatment Comparison		N	S	Z	PROB >[Z]	APPROX SIGNIFIC
Saline vs.	KA	7	29.00	1.7857	0.0741	0.0994
	U-50488H	6	53.00	2.2415	0.0250	0.0466
	KA + U-50488H	6	42.50	0.4890	0.6248	0.6344

Side 2 (Injection Side)

Treatment Comparison		N	S	Z	PROB >[Z]	APPROX SIGNIFIC
Saline vs.	KA	7	21.00	2.9286	0.0034	0.0126
	U-50488H	6	55.00	2.4995	0.0124	0.0295
	KA + U-50488H	6	35.00	0.5604	0.5752	0.5864

Side 1 (Contralateral Side)

Treatment Comparison		N	S	Z	PROB >[Z]	APPROX SIGNIFIC
KA vs.	Saline	6	29.00	1.7857	0.0741	0.0994
	U-50488H	6	21.00	3.9696	0.0030	0.0117
	KA + U-50488H	6	27.00	2.0829	0.0373	0.0593

Side 2 (Injection Side)

Treatment Comparison		N	S	Z	PROB >[Z]	APPROX SIGNIFIC
KA vs.	Saline	6	21.00	2.9286	0.0034	0.0126
	U-50488H	6	21.00	2.9448	0.0032	0.0123
	KA + U-50488H	6	21.00	2.9286	0.0034	0.0126

DISCUSSION

I $^{45}Ca^{++}$ UPTAKE BY RAT SYNAPTOSOMES.

Synaptosomal preparations are an extremely valuable in vitro model to study ion fluxes; results of fundamental importance to the understanding of the kinetics of $^{45}Ca^{++}$ uptake have been published with this preparation (Blaustein, 1975; McGraw, et al., 1982; Adam-Vizi & Ligeti, 1986; Carvalho, et al., 1985). These results have been very informative in understanding the contribution of the voltage-dependent and independent Ca^{++} channels, but it must be kept in mind that synaptosomal preparations represent nerve terminals from an enormous variety of neuronal groups and that these neuronal types may have differing calcium regulating processes (McBurney & Neering, 1987). The method of preparation of the synaptosomes introduces great variability in these types of experiments. The membrane can be made very leaky depending on the material used for the gradient.

Calcium influx through voltage dependant or independent calcium channels is of great importance in the regulation of nerve function and may lead to important secondary changes in nerve excitability and to neuronal degeneration (Miller, 1987; McBurney & Neering, 1987). The calcium uptake due to elevated K^+ is related to membrane depolarization and other mechanisms. At the resting membrane potential in dialyzed axons, it has been found that 70% of the calcium influx was sensitive to tetrodotoxin, 20% was Na^+/Ca^{++} exchange, and the remaining 10% was leakage in dialyzed axons (Requena, 1983). The preincubation time and medium are crucial in determining the final ionic content of

synaptosomes. The activity of the Na^+-Ca^{++} exchange mechanism when the external medium is made high in K+ (K+ substituted for Na^+) is responsible for entrance of Ca^{++} (Carvalho, et al., 1985; McGraw, et al., 1982). Preincubation in a K+-rich medium results in inhibition of the K+-depolarized uptake since neither a normal resting membrane potential nor a Na^+ gradient exists (Carvalho, et al., 1985; McGraw, et al., 1982).

There is disagreement on the terminology used to name the two phases of the calcium accumulation process in synaptosomes. "Fast phase" was defined by Blaustein, (1975); and McGraw, et al., (1982) as a brief period of rapid uptake which is complete within 1-3 sec, while uptake values up to 30 sec were taken to represent the fast phase by Gripenberg, et al,.(1980). We adopted the later definition of fast phase and utilized an 8 sec incubation time unless stated differently.

We found that proveratrine, a Na^+ channel agonist, enhanced Ca^{++} uptake when added to synaptosomes in the incubation medium, both at rest and under depolarizing conditions. Furthermore, verapamil and nifendipine blocked the $^{45}Ca^{++}$ uptake in depolarized synaptosomes. These results are consistent with other published data (Richards, et al., 1984). Thus the synaptosomes utilized in these studies apparently had functional K+, Na^+ and Ca^{++} channels, and their membranes could be polarized and depolarized by the flux of these ions through their respective channels.

The regulation of intracellular calcium is a very complex system not yet fully understood. The participation of pathways with different properties is not straighforward and is presently the subject of intense investigation.

Our data demonstrated the existence of active voltage-sensitive Ca^{++} channels in the synaptosomal preparation. In the presence of external sodium, calcium blockers had no effect on the accumulation of $^{45}Ca^{++}$ in controls, but were able to block a portion of the K+-stimulated uptake.

The Ca^{++} channel agonist, BAY K 8644 did not enhance Ca^{++} uptake in this preparation either in the presence or absence of depolarizing concentrations of K^+. The reason for this is not clear but it suggests that the site of the convulsant action of this compound may not be the nerve terminal Ca^{++} channel (Von Voigtlander, et al., 1987) .

In conclusion, a substantial component of the $^{45}Ca^{++}$ uptake is blocked by two major classes of organic Ca^{++} blockers, dihydropyridines (nifedipine) and phenylalkylamines (verapamil), and by a third group of opiate related compounds: a kappa agonist, U-50488H, and naloxone.

The results showed that U-50488H blocks the influx of $^{45}Ca^{++}$ in K+ depolarized synaptosomes. This effect was seen only in the early phase of the biphasic time-course of K+-stimulated Ca^{++} influx. The early or fast phase is voltage regulated, inactivated by pre-depolarization and low concentrations of La^{++}, and excess K^+ is necessary to open the calcium channels in the presence of a high concentration of Mg++ (Nachshen & Blaustein, 1980). This early phase has been associated with neurotransmitter release (Blaustein & Goldring, 1975; McGraw, et al., 1982). The fast phase of Ca^{++} uptake is inactivated by prior depolarization of the synaptosomes. However, pre-depolarization has no effect on the slow phase of Ca^{++} uptake (McGraw, et al., 1982). Our results are consistent with other studies (McGraw, et al., 1982) and

investigations in electrophysiological systems (Lee & Tsien, 1983) which demonstrated Ca^{++} channel inactivation by depolarization.

The fact that a voltage dependent mechanism is implicated in the blockade of $^{45}Ca^{++}$ uptake by U-50488H is further supported by the decrease of the same magnitude observed with verapamil and nifedipine for the early phase uptake under depolarizing conditions.

The relatioship between Ca^{++} channel blockade and mu opioid receptor mediated mechanisms is unclear. The possibility that opioids inhibit neurotransmitter release by disturbing voltage-dependent presynaptic Ca^{++} uptake during polarization through opioid receptors was suggested by Kamikubo, et al., (1983). Our results failed to show this inhibitory activity of morphine. No effect on K+-stimulated Ca^{++} uptake during the fast or slow phase of uptake at rest was observed with the indicated preincubation times and uptake measurents used in this procedure.

U-50488H has been shown to be unique in its affinity and selectivity for the kappa receptor (Lahti, et al., 1981; Von Voigtlander, et al., 1983; Tang, 1982; Tang & Code, 1983; Mickelson & Lahti, 1985. The benzomorphans, dynorphin A (an endogenous peptide), U-50488H, and its analogs have high affinity for the kappa site. It has been demonstrated that KA-induced seizures alter hippocampal levels of dynorphin as well as Met^5-enkephalin-like (ME-LI) immunoreactivity (Kanamatsu, et al., 1986). A visible decrease in dynorphin A $_{(1-8)}$ in mossy fiber axons within 6 hours after KA was followed by a substantial increase at 48 hours.

The role of these opioid peptides in KA-induced neurotoxicity does not appear to be primary, but they may still play an important role in

modulating neuronal excitability in the hippocampus. It is possible that KA induces a release of enkephalin from the projections of the enthorhinal cortex and dentate granule cells. This release enhances KA-induced neuronal excitability and also induces release of dynorphin, which may act in a compensatory manner to reduce hippocampal excitability (Kanamatsu, et al., 1986). It may be possible that the cytoprotective effect of U-50488H is mediated by stimulating this compensatory mechanism to reduce excitability.

It has been shown that naloxone pretreatment attenuates the convulsions and cell loss induced by high doses of KA, and morphine potentiates seizures and cell loss after intermediate doses of KA (Fuller & Olney, 1979).

The portion of the Na^+-Ca^{++} exchange transport system that is altered by U-50488H is demonstrated by the blockade of K+-stimulated calcium accumulation in the absence of Na^+ ions. The effects of choline substitution for external Na^+ have been studied extensively (Blaustein & Oborn, 1975; Blaustein & Hodgkin, 1969; Blaustein, 1984). This particular type of Ca^{++} channel, the Na^+-Ca^{++} carrier, exchanges one Ca^{++} for three or more Na^+ ions countertransported. Since net charge is moved by the carrier, the transport can be voltage regulated (Blaustein, et al., 1974). The energy is provided by the electrochemical gradient across the membrane. The exchange appears to be symmetric in that Ca^{++} can move in both directions, depending upon the prevailing Na^+ gradient. When the Na^+ gradient is reduced (e.g. by inhibiting Na^+-K+ pump activity and increasing the intracellular Na^+ concentration or by depolarizing the cells), net Ca^{++} influx takes place. In brief, Ca^{++} influx is increased when external Na^+ is reduced

and/or internal Na^+ is increased. Conversely, Ca^{++} efflux from $^{45}Ca^{++}$ loaded synaptosomes is reduced when external Na^+ is lowered (McGraw, et al., 1982). In the present study, K+-stimulated calcium uptake was not apparent in the absence of Na^+. Results by Turner & Goldin (1985) showed increased calcium influx during the first 4 sec. U-50488H did not alter the fast phase component of the calcium uptake by depolarized synaptosomes. In a Na^+-free and K+-rich medium, where neither a membrane potential nor a sodium gradient probably existed in the synaptosomes (Carvalho, et al., 1985), no activity could be observed during the early phase with U-50488H. The contribution to the slow $^{45}Ca^{++}$ uptake which is Na^+-dependent and perhaps altered by U-50488H was not studied.

Demonstration of a component associated with the decrease of $^{45}Ca^{++}$ uptake in depolarized synaptosomes in the presence of U-50488H is not easy at this point. Previously mentioned differences in preparation of synaptosomes, incubation time and medium make interpretation of results difficult. However, the best candidate for an activity of U-50488H will be a mechanism related to long term regulation of neuronal activity, such as the regulation of resting potentials, since the early phase of Ca^{++} uptake is mostly related to secretion of neurotransmiters and our measurements were taken at the end of the early uptake curve. Other mechanisms to explore will be receptor operated channels controlled by calcium binding proteins such as calmodulin, parvalbumin, calbindin and PEP-19. Calbindin appears to be located in hippocampal CA1 pyramidal and dentate granule cells, and calmodulin in the cell bodies and dendrites of most neurons in the CNS (Meldrum, 1984; McBurney & Neering, 1987).

The selective vulnerability of hippocampal neurons during status epilepticus is associated with Ca^{++} movement into the neurons and is attributable to membrane properties that give rise to burst firing and an excessive, sustained entry of calcium (Meldrum, 1981). The events that take place after the entry of calcium may in turn influence other processes such as permeability, activation of peptidases and proteases and exposure of glutamate receptors in the membranes. This will make the neurons more susceptible to excitatory inputs and more likely to show hyperexcitability states (Baudry, et al., 1983). Demonstration of changes in calcium uptake by synaptosomes in the presence of excitatory amino acids and U-50488H will support the theory of the involvement of excitatory amino acids in the pathology of epilepsy and the cerebroprotective effects of drugs that will act on this mechanism. No significant differences were observed in the mean values for $^{45}Ca^{++}$ uptake by synaptosomes at rest or under depolarizing conditions when measured in the presence of NMDA or kainic acid. However, there was a significant decrease when glutamate was added to the system at rest and in depolarizing buffer. The presence or absence of Mg++ did not influence this decrease of $^{45}Ca^{++}$ uptake by the synaptosomes.

The contribution of the mitochondria as a buffering system for intracellular calcium may be an interesting process to investigate in association with the blocking activity of U-50488H. This system has an extremely high capacity for Ca^{++} and stores it in an inert form as $Ca(PO_4)_2$. This energy-linked process can be supported by either electron transport or by the hydrolysis of ATP. It successfully buffers the massive calcium influx into hippocampal neurons associated with seizure activity (Griffiths, et al., 1984). It may be associated with

the terminal cisternae of the ER as calcium sequestering organelles, providing a direct pathway for the transport of Ca^{++} from within the neuron to the extracellular space (McBurney & Neering, 1987). More studies will be necessary to reveal the exact site of action of U-50488H in the complex scheme of calcium-dependent processes affecting neuronal activity.

II (^{3}H)KA BINDING ASSAY

The purpose of using this binding assay was to evaluate the effect of U-50488H as a displacer of (^{3}H)KA binding under different ionic conditions. The role of excitatory amino acids in regulating neuronal excitability is not well defined. Evidence is accumulating suggesting that a mechanism involving elevation of intracellular Ca^{++} is important in the regulation of synaptic plasticity (Morris, et al., 1986) and in mediating some pathophysiological effects of amino acid excitotoxicity (Choi, 1985). The anticonvulsant action of NMDA receptor antagonists highlights the role of NMDA receptors in regulating excitability (Meldrum, 1985). Even though l-glutamate and related acidic amino acids are firmly established as major excitatory synaptic substances in vertebrate CNS, the characterization of these receptors is still at an early stage. Selective antagonists are not available and KA and quisqualate are not as specific as originally thought. These facts obscure the determination of the precise mechanism of action of these substances and their analogues and antagonists at the excitatory synapses.

The role of NMDA and non-NMDA receptors in the convulsant or anticonvulsant effects related to epilepsy is not known. However, it is believed that more than one type of receptor for excitatory amino acids is engaged in seizures, irrespective of the receptors preferentially activated in the initiation of the convulsions.

In order to draw meaningful conclusions in terms of receptors involved in the physiological events underlying seizures, the availability of individual antagonists is essential. U-50488H antagonizes seizures induced by both KA and NMDA (Von Voigtlander, et al., 1986). This observation is of great interest in view of the suggestion that the voltage-dependent behavior of conductance activated by NMDA receptors may modulate responses induced by non-NMDA receptors (Majer & Westbrook, 1984). U-50488H anticonvulsant effects suggest that the same ionic mechanisms underlying the seizures are being protected. The (^{3}H)KA binding assay may be used as a tool for the development of anticonvulsant drugs. Compounds which displace the binding of radiolabeled excitatory amino acids from their receptors might be excitatory amino acid antagonists and anticonvulsants.

In the absence of highly selective antagonists, the antagonism of KA induced seizures by U-50488H provides us with a new chemical structure which might prove to be a displacer of KA binding. (^{3}H)KA was used in this study due to the previously described hypothesis of kainate and quisqualate receptors as primary mediators of rapid excitatory signals at acidic amino acid synapses (Fagg, 1986).

Assay conditions were similar to those described by London and Coyle (1979). Endogenous glutamate was released by an incubation of 30

min at 37 C and subsequent washing and centrifugation of the synaptic membranes.

The results of the present study showed that only one population of receptors was detected by this ligand binding technique when assayed in a Ca^{++}-free buffer. Other studies have shown the presence of two sites (London & Coyle, 1979; Simon, et al., 1976; Unnerstall & Wamsley, 1983; Monaghan & Cotman, 1982; Tremblay, et al., 1985; Honore, et al., 1986) in the rat brain. The rationale for using mice was to be able to correlate binding with the physiological and morphological studies. However in the mouse brain study in the mouse brain, performed in the presence of Cl^- and Ca^{++} ions the linear model for one site was automatically rejected by the statistical package on the basis of the F test and the Akaike information criteria (ACI).

Assay precision was high when determinations were made in the Ca^{++}-free system, whereas precision in the presence of (Ca^{++} 2.5 mM) was not as good. This lack of precision in the (^{3}H)KA binding assay could be attributed to the washing method which might have introduced significant error by disrupting the binding sites.

There are two findings in this study that support the hypothesis that U-50488H is involved in a Ca^{++} related anticonvulsant mechanism. First is the finding that the affinity (K_d) of the KA receptors was decreased in the presence of Ca^{++} (2.5 mM). Second is the finding of the decrease of the population of receptors (B_{max}) in the presence of U-50488H, Ca^{++}, and Cl^-. Previous experiments by Rothman (1985) suggested that the pathophysiology of amino acid neurotoxicity may be due to the passive distribution of Cl^- ions under depolarizing conditions. An increase in intracellular Cl^- concentration cannot be

compensated by outward anion flux, because the bulk of intracellular anions are impermeant. Therefore, more cations (i.e.Ca^{++}) are drawn into the cell, which increases intracellular osmolarity and water enters the cell. Lysis follows after excessive tension in the cellular membrane.

Calcium has been associated with the phenomena responsible for neuronal lysis during hypoxic injury, seizure activity and spinal cord contusion (Siesjo, 1981). Release of glutamate is enhanced by Cl^- and Ca^{++} (Nadler, et al., 1985) and may be responsible for the neuronal damage observed under these conditions. Kainate produces edema, astrocytosis and reduction of protein content. The accumulation of Ca^{++} undergoes dramatic changes after kainate. This is possibly associated with the binding of this cation to macromolecules or molecular aggregates formed or released by the disintegrating neural elements (Korf & Postema, 1984). The mechanism underlying the protective effect of U-50488H is not clear, though a marked reversal of the affinity of the kainate receptors in the presence of Ca^{++} and Cl^- ions is evident in the presence of this compound.

III (^{3}H)-DG UPTAKE IN VARIOUS MOUSE BRAIN REGIONS.

Radiolabeled 2-deoxyglucose has been widely used to measure regional metabolic rates in the brains of laboratory animals (Sokoloff, et al., 1977). The theoretical basis of this technique rests on the facts that neurons use glucose as their main source of energy, and hyperactivity states lead to an increase of energy expense. The radiolabeled deoxyglucose is administered intravenously as a pulse,

which is then taken up by the neuron and phosphorylated as if it were normal glucose, but is not further metabolized. The labeled 2-DG is trapped inside the cell in concentrations proportional to the integrated uptake of glucose. Liquid scintillation counting of the solubilized areas of the brain provide a measure of local activity. This type of experiment was relatively easy to prepare and a rapid determination of a trend could be obtained indicating an effect of U-50488H on neuronal metabolism. A potential disadvantage that was observed was that the standard error was large, and attempts to correlate the data may be obscured by the high variation between mice. (^{3}H)DG was used in this study to follow the distribution of labeled material following pretreatment with KA and U-50488H. The conditions for assaying changes in mice were similar to those reported by Von Voigtlander, et al., (1983). This semiquantitative method was used to evaluate the metabolic alterations in structures which are known to be associated with epileptiform activity (hippocampus, corpus striatum and septum).

Other studies using ^{14}C-DG have shown the effects of KA after systemic administration; the brain area affected primarily was the limbic system (Ben-Ari, et al., 1981). The present results similarly demonstrate enhanced glucose utilization after i.c.v. KA.

The role of excitatory amino acids in the brain damage observed after seizures is not fully understood. Metabolic and electrographic changes have provided evidence that the hippocampus, the amygdala and other limbic structures occupy a central position in this syndrome. Autoradiographic deoxyglucose maps revealed an increase in glucose consumption exclusively in the hippocampal formation and lateral septum

(Ben-Ari, et al., 1981) in less than 1 hr after systemic administration of KA. At longer delays, structures receiving hippocampal projections are progressively involved, showing a remarkable correlation between electrographic changes and the rise of metabolism. Furthermore, the signs of degeneration observed were almost exclusively located in structures in which there had been a rise in metabolism (Ben-Ari, et al., 1981).

The results of this study showed that no significant changes were produced in the uptake of 2DG in the hippocampus, corpus striatum or septum of mice with U-50488H, nor did U-50488H did block the effects of KA detectable with this procedure.

In contrast, standard anticonvulsants such as diazepam and phenytoin showed a marked inhibition of the increased glucose metabolism induced by KA in the limbic system. Metabolism was also decreased when the drugs were administered by themselves. Thus, the anticonvulsant effect of these compounds may be independent of the excitatory amino acid mechanism. It is suggested that the mechanism of action of U-50488H may be related to other aspects of the seizure syndrome but not to metabolic changes. The similarities between the effects of KA and of (D-Ala^2, D-Leu^5) enkephalin (DADL), an opioid peptide (Aitke, 1985; Routtenberg, et al., 1984), are suggestive of a mechanism involving release of an endogenous opioid from mossy fibers as a normal mechanism for enhancement of CA3 pyramidal cell excitability. This endogenous opioid could be dynorphin. U-50488H's effect as an anticonvulsant may be mediated through such an opioid-related mechanisms.

IV EFFECTS OF U-50488H ON KAINIC ACID INDUCED LESIONS IN THE MOUSE HIPPOCAMPUS. A MORPHOMETRIC STUDY BY IMAGE ANALYSIS

The excitatatory amino acids have been suggested to be the common pathogenic factors in epilepsy, hypoglycemia and cerebral ischemia. Differences in the distribution of damage have been explained by differences in the site of excitatory amino acid release, type of receptor activated, or secondary intracellular reactions such as acidosis (Siesjo & Wieloch, 1986).

The purpose of this study was to obtain a quantitative measurement of the effects of U-50488H pretreatment on the area of vacuolization induced by KA in the hippocampus. The image analysis method permits the assessment of local neuronal damage in brain regions by measuring the area of vacuolization. In this study, attention was restricted to a region of the hippocampus, the CA3 area, selected on the basis of the presence of lesions after i.c.v. KA administration.

An <u>in vivo</u> study of the anticonvulsant properties of U-50488H was undertaken using a small animal model, where recovery was allowed following the establisment of KA-induced lesions. We observed the density and distribution of neuronal damage in the hippocampus in control and U-50488H pretreated mice after a 4 day recovery. The design of this study overcame the shortcomings limiting the traditinal subjective interpretation of lesions by light microscopy. It provided us with a procedure of high resolution to study the cytoprotective activity specifically related to the anticonvulsant activity of

U-50488H. The neuronal damage quantified by measurement of the area of vacuolization was used as a marker for neuronal protection.

The seizure pattern produced by i.c.v. KA was similar to that described earlier in rats (Ben-Ari, 1981; Nitecka, et al., 1984). These and other observations suggest that this is a good model of temporal lobe epilepsy. The determination of long term sequelae in this animal model, notably in respect to the prevention of the pathological processes, may contribute to the understanding of the anticonvulsant and cytoprotective effects of U-50488H.

Convulsive seizures occurred twenty minutes after i.c.v. injections of KA, but not all mice treated with KA showed overt seizures. The animals observed for 1 hr after the neurotoxin injection displayed general agitation, forepaw tremors, salivation, jumping and tonic convulsions. Mice pretreated subcutaneously with 100 mg/Kg U-50488H did not show any of these abnormal behaviors. Survival was 100% for both groups at the end of the 4 days.

Prominent histopathological alterations were observed in the CA3 region of the hippocampal formation of the KA treated group. The alterations included shrinkage of tissue, progressive degeneration of neurons, necrosis associated with cell loss and proliferation of glial elements. Pathological alterations of the CA1 subfield were rare and restricted to a very small area when found. No damage was observed in other areas of the brain under general examination of the tissues. The image analysis was restricted exclusively to the CA3 area of the hippocampus.

The histopathological changes observed in the animals pretreated with U-50488H before neurotoxin administration were qualitatively

similar to those observed in the KA treated controls. However, the cellular loss was markedly reduced and the area of vacuolization found was significantly decreased.

The assumption in current efforts to localize the histopathological changes in kainic acid seizure-brain damage syndrome is that the neuronal loss and the development of gliotic scars, especially in limbic structures following KA administration, is reminiscent of that seen in human temporal lobe epilepsy (Ben-Ari, 1985). Investigations on KA effects have examined whether the reported brain lesions following intracerebroventricular KA were secondary to the seizures induced by this neurotoxin or a direct action of this convulsant agent. Pretreatment with diazepam reduced hippocampal damage without reducing the toxic effects of KA at the site of the injection. These results suggest that the distant brain lesions may be selective and secondary to the sustained epileptiform activity (Ben-Ari, et al., 1979). The severe cytotoxic brain edema reflected by the massive swelling of perineuronal and perivascular astroglia may result in disturbance of the local microcirculation and may play a very important role in the pathogenesis of cellular damage. This can be regarded as a local non-selective damaging process which may have an important role in the development of neuropathological alterations. The selective seizure-related damage is accompanied by calcium accumulation or zinc release (Sztriha, et al., 1986). The selective effects of KA are influenced by anticonvulsant drugs such as diazepam while the non-selective effect is prevented by mannitol (Lassmann, et al., 1984).

The role of different types of edema in cell death associated with epilepsy is not clearly understood. Vasogenic edema may contribute to cell damage observed in thalamus but not to that one observed in the hippocampus. Dexamethasone has been shown to protect against vasogenic brain edema in the thalamus but it does not influence the cytotoxic damage observed in the hippocampus. The role of newly synthetized protein(s) with antiphospholipase A_2 activity are suggested in the anti-edema action of dexamethasone (Ruth, 1984; Sztriha et al., 1986).

The results of this study suggest that U-50488H has a protective effect due to its actions on two different kinds of mechanisms. The first is the anticonvulsant component of the effect, which may be related to its ability to antagonize seizures elicited with KA, other excitatory amino acids and BAY K 4688. This effect is probably related to calcium but may be also related to the ability of U-50488H to antagonize the discharges produced in the CA3 region of the hippocampus and the recurrent activation of the amygdalohippocampal axis. A high density of KA binding sites has been found in the stratum lucidum of the CA3 region of the hippocamus, which has been shown to be the most vulnerable structure to the excitatory and cytotoxic effects of KA (Berger & Ben-Ari, 1983). The excitatory amino acids are linked to mixed ionic mechanisms involving Cl^-, Ca^{++}, Na^+, and K^+ (Mayer & Westbrook, 1985) but the precise nature of the permeability changes caused by these compounds remains unclear. The second is the anti-edema effects of U-50488H, which have been shown to prevent increases in water, Na^+ and K+ content (Silvia, et al., 1987); these effects seem to

be related to a reduction of circulating ADH as well as to a direct effect on the kidney (Slizgi & Ludens, 1982).

However, the importance of this antiedema property to the present observations must be questioned as U-54494A, which shares the anticonvulsant mechanism of U-50488H (Von Voigtlander, et al., 1987[b]) and blocks KA lesions (Camacho Ochoa, et al., 1987), does not have water diuretic or antiedema properties (Ludens & Tang personal communications). More experimentation will be necessary to completely elucidate the cerebroprotective effects of U-50488H.

CONCLUSIONS

Effects of U-50488H on $^{45}Ca^{++}$ uptake by rat synaptosomes, (^{3}H)KA binding to mouse synaptic membranes, and antagonism of KA-induced lesions in the CA3 region of the mouse hippocampus support our hypothesis that the cerebroprotective effect of this compound is associated with a calcium-related mechanism underlying seizures.

The $^{45}Ca^{++}$ uptake data were obtained from classical synaptosomal preparations purified in Ficoll gradients. The values observed indicated that U-50488H decreased the increased Ca^{++} uptake elicited by depolarizing K+ concentrations and that this effect was related to voltage regulated channels.

The preparations used were physiologically responsive to different ions of importance in control of permeability of the neuronal membrane. The variability of these experiments may be due to the size, brain region origin and viability of the synaptosomes isolated with Ficoll. However, the reduction in calcium uptake was evident in the presence of U-50488H.

Binding of (^{3}H)KA to receptor sites on mouse forebrain synaptic membranes was decreased by U-50488H in the presence of calcium and chloride ions. The binding parameters of (^{3}H)KA were determined from Scatchard plots in standard mouse washed synaptic membranes. The results indicated that in the presence of U-50488H the density of KA receptor binding sites decreased, while the K_d values obtained indicated a shift towards higher affinity receptor sites. These changes suggest alterations in the shape of receptor proteins, which may be due to calcium interactions.

No significant differences were observed in mean values for (^{3}H)DG uptake in the presence of U-50488H when compared to kainic acid controls. These findings indicated that the cerebroprotective effects of this compound are not glucose metabolism related. Other metabolic probes will be valuable to facilitate the understanding of U-50488H effects on the hyperexcitable neuron's metabolic pathways. The significant increases of (^{3}H)DG uptake observed with kainic acid in brain areas related to generation of seizures emphasizes the role of excitatory amino acids in the etiology of epilepsy.

Significant decreases in the area of vacuolization induced by i.c.v. KA were observed in the CA3 region of the hippocampus of U-50488H-treated mice when quantitated by image analysis. The decreased area of vacuolization in the lesioned side of the hippocampus indicates that U-50488H previously administered to mice receiving KA protects the pyramidal cells of the CA3 region from excitotoxic lesions.

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FOOTNOTES

FOOTNOTES

a ^{45}Calcium, Amersham, Amersham Corporation, Arlington Heights, Illinois, 60005.

b Calcium chloride, Mallinckrodt Incorporated, Science Products Division, St. Louis, Missouri, 63134.

c Charles River Breeding Laboratories, Inc., Portage, Michigan, 49001.

d Glass-teflon homogenizer, Kontes Glass Company, Vineland, New Jersey.

e Sorvall RC5C Model centrifuge, Sorval Instruments,Bioresearch Systems, Sorvall Products, Wilmington, Delaware, 19898.

f Ficoll 400, Pharmacia Fine Chemicals, Division of Pharmacia Inc., Piscataway, N.J. 08854.

g Beckman Model L2-65 Ultracentrifuge, Beckman Instruments, Inc., Bioanalytical Systems Group, Arlington Heights, Illinois, 60004.

h Sorvall Swinging Rotor SW28, Beckman Instruments, Inc., Bioanalytical Systems Group, Arlington Heights, Illinois, 60004.

i Bio-Rad Protein Assay Dye Reagent Concentrate, Bio-Rad Laboratories, Richmond, Calif. 94804.

j Spectronic 21, Bausch and Lomb, Analytical Systems Division, Rochester, New York, 14625.

k Whatman GF/A filters, Whatman International Ltd., Maidstone, England.

l ACS* Aqueous Counting Scintillant,Amersham Corporation, Arlington Heights, Illinois, 60005.

m Phillips 201 Electron Microscope, Phillips Electronic Instruments Inc., Mahwah, N.J. 07430.

n Dupont-Sorvall MT 5000 Ultramicrotome, Sorvall Instruments, Bioresearch Systems, Sorvall Products, Wilmington, Delaware, 19898.

o Polybed 812, Polysciences, Inc., Warrington, PA,

p Liquid Scintillation System, Beckman, Scientific Instruments Division,

q Whatman GF/B filters, Whatman International Ltd., Maidstone, England.

r (^{3}H)KA, Amersham, Amersham Corporation, Arlington Heights, Illinois, 60005.

s Kainic Acid, Sigma Chemical Company, St. Louis, Missouri, 63178.

t 2-(^{3}H)-Deoxy-D-Glucose, E.I. du Pont de Nemours & Co., (Inc.), NEN Research Products, Boston, MA 02118.

u Protosol, Du Pont, NEN Research Products, Boston, Massachussetts, 02118.

v Teflon Liners, Du Pont, NEN Research Products, Boston, Massachussetts, 02118.

w U-50488H, The Upjohn Company, Kalamazoo, Michigan, 49001.

x Methoxyflurane, Metofane, Pitmann-Moore, Inc., Washington Crossing, N.J. 08560.

y Microtome, Leitz 1512, Leitz Leica Wild Heerbrugg, W. Nuhsbaum, Inc. Mc Henry, Illinois.

z Image analizer, Joyce-Loebl Magiscan 2A, Nikon Inc., Instrument Group. 623 Steward Ave, Garden City, NY, 11530.

aa Brinkman Homogenizer, Brinkman Instruments, Westbury, NY, 11590.

ab Rodent Laboratory Chow #5001. Purina Mills, Inc.

ac Veralba (Proveratrines A and B) Pitman-Moore, Division of the Dow Chemical Company, Indianapolis, In.

APPENDICES

APPENDIX 1 - Assay Buffers

A. 0.32 M sucrose 5 mM HEPES buffer pH = 7.4

	Gram/Liter
Sucrose	109.54
HEPES (Na salt)	1.30
EGTA	0.19

B. Density gradient solutions

1. Ficoll 4%

	Grams/Liter
Ficoll	40.0

then add 0.32 M sucrose to q.s. 1 liter

2. Ficoll 11%

	Grams/Liter
Ficoll	110.0

then add 0.32 M sucrose to q.s. 1 liter

APPENDIX 1 continued

C. Incubation medium

1. 5.6 mM KCl 20 mM TRIS buffer pH = 7.4

	Concentration	Gram/Liter
NaCl	136.0 mM	7.95
KCl	5.6 mM	0.42
$MgCl_2$	1.3 mM	0.26
TRIS/HCl	20.0 mM	2.42
Glucose	11.0 mM	1.98
$LaCl_3$	0.5 mM	0.19

2. 74.4 mM KCl 20 mM TRIS buffer pH = 7.4

	Concentration	Gram/Liter
NaCl	67.2 mM	3.93
KCl	74.4 mM	5.55
$MgCl_2$	1.3 mM	0.26
TRIS/HCl	20.0 mM	2.42
Glucose	11.0 mM	1.98

APPENDIX 1 continued

3. 40.0 mM KCl 20 mM TRIS buffer pH = 7.4

	Concentration	Gram/Liter
NaCl	67.2 mM	3.93
KCl	74.4 mM	5.55
$MgCl_2$	1.3 mM	0.26
TRIS/HCl	20.0 mM	2.42
Glucose	11.0 mM	1.98
$LaCl_3$	0.5 mM	0.19

APPENDIX 2 Incubation buffers

1. 50.0 mM TRIS Buffer pH = 7.1

	Concentration	Gram/Liter
TRIS/Acetic Acid	50.0 mM	6.06

2. 50.0 mM TRIS/ HCl buffer pH = 7.1

	Concentration	Gram/Liter
TRIS/HCl	50.0 mM	6.06

APPENDIX 3 - 2-(^{3}H)-DG Solution

A. (^{3}H)DG solution

	ml
(^{3}H)DG stock	0.5
saline	9.5

APPENDIX 4 - Mouse Diet

A. Contents of mouse maintenance diet (*)

Crude protein not less than	23.0%
Crude fat not less than	4.5%
Crude fiber not more than	6.0%
Ash not more than	8.0%
Added minerals not, more than	2.5%

(*) Rodent Laboratory Chow #5001. Purina Mills, Inc.

APPENDIX 5 - Fixative

B. Karnovsky's fixative

	Concentration	
Paraformaldehyde		20.0 gm
Glutaraldehyde	50%	50.0 ml
Na Cacodylate	0.2 M	500.0 ml
NaOH	1.0 N	dropwise until clear solution

VITA

Marlene Camacho Ochoa was born in Armenia, Colombia on March 11, 1943 and graduated from Colegio Oficial Santa Teresa de Jesús in 1960. She received the Químico Farmacéutico degree in the Facultad de Ciencias, Universidad Nacional de Colombia in 1966 and the Diplôma d'Études Approfondies from Université de Paris (France) in 1969. She was Assistant, Associate Instructor and Assistant Professor at the Department of Physiology, Pharmacology and Biochemistry, Facultad de Medicine, Universidad Nacional de Colombia from 1966-1983. In 1978 she was accepted into the graduate program of the Department of Veterinary Physiology, Pharmacology and Toxicology, School of Veterinary Medicine, Louisiana State University, as a graduate student. She joined the CNS Diseases Research Unit of The Upjohn Company in 1982.

DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Marlene Camacho Ochoa

Major Field: Veterinary Medical Sciences (Toxicology Option)

Title of Dissertation: Cerebroprotective Activity of U-50488H: Relationship to Interactions With Excitatory Amino Acids and Calcium

Approved:

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

Date of Examination:

November 13, 1987